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A NODULATION-INHIBITING ROOT EXCRETION FROM A NON-NODULATING SOYBEAN STRAIN[1]

GERALD H. ELKAN[2]

Abstract

A non-nodulating, near-isogenic soybean strain has been shown to release, during growth, a substance that inhibits nodulation of its normally nodulating sister strain. In greenhouse experiments, inoculated seedlings of both strains were transferred to vessels containing aerated, nitrogen-free, Hoagland's nutrient solution. Two plants per vessel were established in the following combinations: one nodulating and one non-nodulating, two nodulating, or two non-nodulating. Root excretions from the mutant resulted in highly significant decreases in nodulation of the normal plants. In addition, presence of the mutant also resulted in decreased total nodule weight, total dry weight, and total nitrogen per nodulating plant. Nodulation in ladino clover, when grown in nutrient solution, was also significantly inhibited by the presence of the mutant soybean. The excretion apparently does not inhibit growth of *Rhizobium japonicum*.

Introduction

A potentially valuable tool for the investigation of factors governing nodulation is available because of the development of a near-isogenic non-nodulating soybean line. Comparative studies using this mutant and the nodulating sister strain offer a possible means for the determination of specific nutritional and environmental factors critical to nodulation.

The evidence indicates that physiological interrelationships between the legume host and the rhizobia are involved in the mechanisms of infection. Wilson (10) reported the results of several experiments indicating that the roots of legumes excrete materials which increase the number of rhizobia in the rhizosphere. Similarly, Fred *et al.* (2) reported the results of several investigators which showed that the growth of plants resulted in varying

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numbers of rhizobia in the rhizosphere, and that the growth of certain plants, notably legumes, resulted in large numbers of rhizobia in the root zone. Korenyako (4) showed that the root excretions of some plants stimulated, while others inhibited, the growth of rhizobia in the rhizosphere.

In addition to increasing the numbers of rhizobia, the host roots excrete substances that stimulate the infection process. Thornton (6) observed that no infection occurred during the 8-day lapse between germination and the opening of the first leaf of alfalfa, whereas 1 day later 2% of the root hairs were infected. He attributed this lag to delayed production of substances which stimulated the infection. Nodulation of younger plants was stimulated by the addition of the solution surrounding the roots of older plants. In another work, Thornton (7) showed that the stimulatory substances were not produced in the tops of the plants since the removal of the leaves or stems did not appear to delay nodulation. Nutman (5) grafted genetically resistant clover roots to the tops of susceptible plants and further corroborated the evidence that the factors responsible for resistance to rhizobial infection were localized in the roots and not controlled by the stems or tops. Moreover, nodules have been produced on excised roots, further indicating that the root plays a more important role than the stem.

Both inhibitory as well as stimulatory excretions may be formed by the same plant. Turner (8) and Vantsis and Bond (9) reported that activated charcoal added to the rooting medium of clover inoculated with effective strains of rhizobia leads to a stimulation of nitrogen fixation. Addition of the eluate from the charcoal resulted in inhibition of nodulation.

Clark (1) compared nodulation responses and several other characteristics of a near-isogenic, normally non-nodulating soybean line with a nodulating sister line. Roots of both lines were found to harbor equal numbers of rhizobia, irrespective of the degree of nodulation. Grafting experiments confirmed that the nodulation responses were determined by the rootstocks.

Materials and Methods

The plant material used in this study consists of the F_5 generation of a cross between Lee, a commercial soybean variety in North Carolina, and L9-674, a non-nodulating variety developed at Illinois. These crossings resulted in a nodulating strain, NC N 58-5253 (designated *NN*), and a segregating, near-isogenic, non-nodulating strain, NC N 58-5259 (designated *nn*).

Solution culture methods were utilized to ascertain the effects of root excrements of both soybean strains upon subsequent nodulation. Half-gallon polyethylene freezer containers with polyethylene covers were painted and prepared as growth vessels for use in greenhouse experiments. Two holes, approximately 3/4 cm in diameter, were drilled into the cover near opposite corners and a 1-in.-long piece of polyethylene hose inserted in each hole to support the soybean plants. A smaller hole was drilled in another corner of the cover to allow placement of a capillary tube to supply aeration to the nutrient solution. This aeration was controlled with screw clamps on the rubber hose leading from the capillary tube to the main air manifold. Each container was filled with nitrogen-free Hoagland's (3) nutrient solution, plus a small amount of oyster shell for pH control. For experimental purposes,

there were established in each of these vessels either two nodulating plants; two non-nodulating plants; or one nodulating and one non-nodulating plant. Since the non-nodulating plants had no nitrogen source, a small amount of nitrate (less than 20% of the amount recommended for complete Hoagland's medium) was maintained in all containers after stored supplies were depleted. This, naturally, had the effect of reducing nodulation by about one-third in the nodulating strain.

These experiments were designed to answer the following questions: Are substances stimulatory to nodulation excreted by the roots of the nodulating plant? Or, do these root excretions contain substances missing in the excretion of the non-nodulating plants? If so, growing both strains in the same container with their roots in close contact could stimulate some nodulation in the non-nodulating strain. Conversely, inhibitory substances excreted by the non-nodulating mutant, under similar conditions, might inhibit nodulation in the normally nodulating soybean.

In the first set of experiments, seeds of both soybean strains (*NN* and *nn*) inoculated with *Rhizobium japonicum* were allowed to barely germinate and then transferred to the polyethylene containers of nutrient solution as described above. Each container had either two *NN* plants or one *NN* and one *nn* plant. Nodule numbers were obtained periodically.

Another study was designed to see whether accumulation of root excretions in the nutrient solution would influence nodulation. Single, inoculated, *NN* or *nn* plants were allowed to grow in 50 containers of nutrient solution before a freshly germinated, inoculated *NN* seedling was introduced into each container. The nodulation of these latter *NN* plants was followed.

In another study, uninoculated seeds were germinated in vermiculite and inoculated at the time they were transferred to the nutrient solution. Pairs of *NN*, or *NN* and *nn*, plants were placed in each vessel. The *NN* plants were analyzed 30 days after inoculation for nodule weight, total dry weight, and total nitrogen per plant.

In order to determine whether the nodule-inhibiting factor(s) is specific for the strain of soybean used, or whether it might be of more widespread importance, screening experiments were conducted using various legumes and growing them in association with the non-nodulating mutant. The combinations in these experiments were as follows: two of the legumes to be tested grown in association in Hoagland's solution; the test legume plus nodulating soybean; or the test legume and non-nodulating soybean. Duplicate studies were conducted testing ladino clover and alfalfa, and a single trial testing garden beans. In each of these experiments the two soybean strains and the test legume seedlings were germinated and inoculated with the proper rhizobia at the time of transfer to the nutrient solution.

Results and Discussion

It was observed that, when inoculated seeds of the nodulating plants were transferred to the nutrient solution, a statistically significant decrease ($P < 0.01$) in subsequent nodulation was attributable to the presence of the non-nodulating strain. As summarized in Table I, 12 days after inoculation of the seeds, *NN* plants grown in association with other *NN* plants averaged

37 nodules per plant, while the *NN* plants grown in association with *nn* plants averaged only 22 nodules, a reduction of 40.5%. The inhibitory effect persisted for 53 days, the duration of the experiment. In two other such experiments similar, statistically significant decreases in nodulation were measured. Neither in this, nor in the subsequent studies, were any nodules observed on the *nn* plants.

TABLE I

Nodulation of *NN* soybeans from inoculated seeds grown in combination with *NN* or *nn* plants; plants transferred to nutrient solution upon germination

Days after inoculation	Nodule numbers		% decrease
	NN + *NN* combination	*NN* + *nn* combination	
12	37	22	40.5**
24	31	21	32.3**
53	31	19	38.7**

**Significant at the 1% level of probability. $N = 16$.

If the presence of the *nn* plant is inhibitory to nodulation of an *NN* plant grown in association, the inhibitory action might be intensified by allowing the excretion to accumulate. The results of a study to test this hypothesis are summarized in Table II. In 50 vessels, one *NN* or *nn* plant was established. After 10 days *NN* seedlings were inoculated and one transferred to each of the flasks. As is seen in the table, presence of the *nn* plant resulted in statistically highly significant decreases in nodulation of the *NN* seedlings, although this inhibition was not as great as that observed in the first series of experiments. Moreover, over-all nodulation of these delayed planted *NN* seedlings was poor regardless of the association.

TABLE II

Nodulation of seedling-inoculated *NN* soybeans grown in nutrient solution, in combination with *NN* or *nn* plants 10 days older

Days after inoculation	Nodule numbers		% decrease
	NN + *NN* combination	*NN* + *nn* combination	
21	11	8	27.3**
42	13	9	30.8**
50	13	9	30.8**

**Significant at the 1% level of probability. $N = 50$.

In the experiment summarized in Table III, seedling-inoculated *NN* plants again were grown in association with either *NN* or *nn* plants and the presence of the mutant again caused inhibition of nodulation in the *NN* soybean. In this experiment criteria other than numbers of nodules were measured. A statistically significant decrease ($P < 0.05$) in the total nodule weight (19.2%) per *NN* plant was attributed to the presence of the non-nodulating mutant. Significant decreases ($P < 0.05$) in total dry weight

TABLE III

Interaction of *NN* and *nn* soybean plants
(sampled 30 days after inoculation)

Plant combination	Nodule weight, g/*NN* plant	Total dry weight, g/*NN* plant	Total nitrogen, mg/*NN* plant
NN + *NN*	0.0598	0.9606	41.15
NN + *nn*	0.0483	0.6850	29.96
% decrease	19.2*	28.7*	27.2*

*Significant at the 5% level of probability. $N = 26$.

per plant (28.7%), and total nitrogen per plant (27.2%), were observed for *NN* plants in this association.

The evidence at this point indicated that a factor (or factors) excreted by the non-nodulating soybean mutant is able to cause inhibition of nodulation in a normally nodulating, near-isogenic sister strain. In order to determine whether this inhibition by the *nn* plant could also be induced in other legume species, screening experiments such as the one summarized in Table IV were conducted. Statistically highly significant decreases ($P < 0.01$) in nodulation (52%) resulted when ladino clover was grown in association with the *nn* soybean as compared with the nodulation of clover when grown with the nodulating soybean or clover. When this experiment was repeated, a statistically significant decrease ($P < 0.01$) in nodulation of 56.4% was measured. In both experiments slightly better nodulation was observed in the clover – *NN* soybean combination as compared with the clover–clover association. However, these differences were not statistically significant.

TABLE IV

Interaction of ladino clover and non-nodulating soybean plants
grown in nutrient solution
(counted 20 days after inoculation)

Combination	Number of nodules per clover plant	% decrease clover + *nn* vs. clover + *NN*
Clover + clover	4.1	
Clover + *NN* soybean	5.0	
Clover + *nn* soybean	2.4	52**

**Significant at the 1% level of probability. $N = 30$.

Table V summarizes a similar experiment, testing the effect of the non-nodulating soybean upon nodulation in alfalfa. In two such experiments, approximately 16% decrease in nodulation resulted when alfalfa was grown with *nn* soybeans. These results were not statistically significant although they were reproducible. The only other legume tested similarly to date has been string bean (*Phaseolus vulgaris*) and no inhibition of nodulation was observed. Other legumes are presently being screened.

Various experiments have shown that the factor does not inhibit the growth of rhizobia. When various concentrations of filtered root excretion from either

TABLE V

Interaction of alfalfa and non-nodulating soybeans grown in nutrient solution

Days after inoculation	Number of nodules per alfalfa plant			% decrease Alf. + *nn* vs. Alf. + *NN*
	Alf. + Alf.	Alf. + *NN*	Alf. + *nn*	
13	8.2	8.6	7.4	13.9
2	8.3	8.8	7.4	15.9
30	8.5	8.3	7.0	15.7

$N = 42$

the *NN* or the *nn* plant were added to tubes of yeast extract – mannitol broth and subsequently inoculated with rhizobia, no differences in the growth curves of the organisms were observed with comparable concentrations of the two excretions. Water extracts of ground root material from the *nn* plants, also, resulted in no inhibition of growth of rhizobia. Chemically sterilized seeds of both soybean strains were submerged in heavily inoculated pour plates of yeast–mannitol agar and the plates were incubated. No clear zones were found around the seeds or, later, the germinating seedlings, indicating no effect on rhizobial growth.

Rhizosphere populations of both strains of soybeans were studied and these will be published in a later paper. These studies indicate that the number of rhizobia in the rhizosphere of the *nn* plant may indeed be higher early in the growth cycle of the plant. It was further shown that although the two strains differ with respect to only one gene, they contain completely different rhizosphere populations, both numerically as well as nutritionally.

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INHIBITION OF FUNGAL GROWTH BY BACTERIA DURING CELLULOSE-DECOMPOSITION[1]

Y. Henis, Paulina Keller, and A. Keynan

Abstract

The cellulose-decomposing flora developing from soil crumbs on filter paper placed upon an agar medium was studied. Under these conditions fungal development was significantly inhibited by bacterial growth. When cellophane was substituted for filter paper, no inhibition of fungal growth was observed. A pure culture of *Cellvibrio* inhibited the vegetative growth of *Stachybotrys* when inoculated simultaneously on media containing carboxymethyl-cellulose, filter paper, or glucose up to 0.1%. This inhibition was shown to be caused by competition for available carbohydrates.

Introduction

Although the ability to decompose cellulose is widespread among many groups of soil bacteria and fungi, a rather specific cellulose-decomposing flora has been described under specified conditions by different workers. For example, *Cellvibrio* and *Cytophaga* were shown by Winogradsky (14) to predominate in the microflora developing around soil crumbs placed on silica-gel plates covered with filter paper. Stanier obtained inhibition of fungal growth and development of cellulolytic bacteria while using water-saturated filter paper placed upon mineral agar (11). On the other hand, Tribe's investigations, in which glass slides covered with cellophane or filter paper were buried in the soil, showed that cellulolytic fungi were the first organisms to attack cellulose in the soil (13).

The contradictory findings of these investigators may be explained by differences in physical conditions (pH, oxygen tension, temperature, humidity) or differences in the type of cellulose (3). The inhibition of fungal growth on agar plates, however, may also be the result of the competition for nutrients between bacteria and fungi or of the production of antibiotic or toxic substances.

The present paper describes investigations on the effect of some of these factors on the selection of the cellulose-decomposing flora on agar and liquid media.

Materials and Methods

Soils.—Unless otherwise stated samples of brown-red sandy soil (pH 7.2) from the garden of the Faculty of Agriculture, Rehovot, Israel, were used. In addition, samples of neutral and slightly alkaline soils taken from various locations in Israel were tested as indicated in the text.

Fungal and bacterial strains.—A cellulolytic strain of the mold *Stachybotrys* and an unidentified species of the bacterial genus *Cellvibrio* were isolated from soil and used as test organisms. Spore suspensions of *Stachybotrys* were

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Contribution from the Faculty of Agriculture, Hebrew University, Rehovot, Israel, and the Israel Institute for Biological Research, Ness Ziona, Israel.

prepared from a 7-day-old culture on yeast dextrose agar. The agar surface was washed with distilled water, and the spores were collected and washed 3 times with 20 ml of distilled water by centrifugation. The population densities of the spore suspensions were determined with a haemocytometer counting chamber. Bacterial suspensions were prepared from 2-day-old cultures grown on agar containing 1% of lactose at 30° C. The suspensions were washed with saline by centrifugation and resuspended to give an optical density of 0.4 at 550 mμ when measured in a Coleman Junior spectrophotometer, using 19×150 mm test tubes.

Basic medium for testing cellulose-degradation and growth relations between fungi and bacteria.—The medium used contained 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgCl_2.6\ H_2O$, 0.01% Bacto yeast extract (Difco), in 1 liter of distilled water, pH 7.0. For a solid medium, 1.5% Bacto agar (Difco) was added. The carbon sources added to this medium were: Whatman filter paper No. 1; cellulose film, Grade P.T. 300;* carboxymethylcellulose (ICI), specific viscosity of 5.6 at a concentration of 1%, or glucose in various concentrations. The cellophane was treated overnight with 0.1 *M* versene at pH 8.0, and then washed thoroughly with distilled water and boiled for 30 minutes.

Determination of fungal growth.—The fungal mycelium was collected from the liquid medium by centrifugation, washed twice with distilled water, and dried in an oven at 100° C to a constant weight. Growth was expressed as mg dry weight/100 ml medium.

Bacterial growth in liquid medium.—This was determined by measuring optical density of bacterial suspensions in a Coleman Junior spectrophotometer at 550 mμ, using 19×150 mm test tubes.

Reducing sugar.—This was determined by the method of Somogyi (10) and Nelson (7), values being expressed as mg glucose/ml.

Estimation of cellulose-decomposition on agar media.—Strips of filter paper or cellophane, 1 × 8 cm, were placed on agar plates and two soil crumbs (average diameter 2 mm) were placed at equal distances on each strip. Cellulose decomposition was estimated by lifting the strips carefully by means of sterile forceps and determining the time required for decomposition to proceed to such an extent that the two portions of the strip on either side of the soil crumb came apart. When pure cultures were tested, suspensions of the organisms were placed on one location in the middle of the strip.

Experimental and Results

A total of 72 strips of filter paper (width 1 cm) and cellophane, respectively, were placed on agar plates, half of which contained 100 μg/ml chloramphenicol (Lepetit). Two soil crumbs were placed on each of the strips (288 soil crumbs in all). The plates were incubated for 11 days at 30° C and tested for cellulose degradation and microbial growth. The results are summarized in Table I. On filter paper in the absence of chloramphenicol growth was predominantly bacterial. On medium with cellophane both with and without the antibiotic, fungi were the dominant organisms. This experiment was repeated

*Kindly supplied by the British Cellophane Co., Ltd., Bridgwater, Somerset, England.

TABLE I

Cellulose degradation and microbial growth around soil crumbs on agar medium

Substrate	Chloramphenicol (100 μg/ml)	Cellulose degradation/growth ratio*	
		Fungi	Bacteria
Filter paper	−	3/3	69/72
Filter paper	+	70/72	0/0
Cellophane	−	66/72	0/4
Cellophane	+	72/72	0/4

* $\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{No. of soil crumbs around which cellulolytic activity was observed}}{\text{No. of soil crumbs with microbial growth}}$.

TABLE II

The effect of chloramphenicol on the degradation of filter paper and microbial growth around soil crumbs on agar medium

Incubation time: 11 days at 30° C

Soil type	Chloramphenicol (100 μg/ml)	Cellulose degradation/growth ratio*	
		Fungi	Bacteria
Loamy sand (20 years of citrus growth)	−	2/2	20/20
	+	20/20	0/0
Loamy sand (9 years of citrus growth)	−	0/0	20/20
	+	20/20	0/0
Soil from a ploughed unirrigated field plot	−	12/12	1/20
	+	20/20	0/0
Alluvial soil, 4 years' irrigation (alfalfa)	−	0/2	17/20
	+	19/20	0/0
Alluvial plain, unirrigated	−	8/12	2/20
	+	6/20	0/0
A virgin sand dune	−	17/18	0/0
	+	18/20	0/0

*As in Table I.

with crumbs of soil samples collected from various locations in Israel. From soil crumbs taken from cultivated and well-irrigated soils and placed on filter paper with chloramphenicol, mainly bacteria developed. Soil crumbs from an uncultivated sand dune showed fungal growth both on filter paper with and without the antibiotic (see Table II).

Most of the cellulolytic bacteria which grew on the filter paper strips belonged to the genus *Cellvibrio*, while some decomposed cellulose in a manner typical of myxobacteria. Some of the cellulolytic fungi were identified as *Stachobotrys*, *Aspergillus*, *Fusarium*, *Curvularia*, *Cephalosporium*, and *Chaetomium*.

The results described above could be explained by the antagonistic action of cellulose-decomposing bacteria towards cellulolytic soil fungi when filter paper was used as the sole carbon source. To test this hypothesis, experiments with pure cultures were carried out.

Pieces of filter paper 1.5 × 1.5 cm were placed upon 12 ml agar in a Petri dish (5 squares on each plate). Twenty pieces were inoculated by means of a micropipette with spores of *Stachybotrys* (2.5×10^3 spores in 0.01 ml on each piece) and 20 with both *Stachybotrys* and *Cellvibrio*. The plates were incubated at 30° C and observed by direct microscopy for fungal growth and sporulation. All pieces inoculated both with *Stachybotrys* and *Cellvibrio* showed a very limited mycelial growth and no sporulation, whereas pieces inoculated with *Stachybotrys* alone showed mycelial growth after 24 hours and sporulation after 48–72 hours. When the experiment was repeated with cellophane instead of filter paper, the growth of *Stachybotrys* was not inhibited by *Cellvibrio*. This may result from the limited growth of *Cellvibrio* on cellophane, while filter paper placed on the same solid agar medium was readily decomposed by *Cellvibrio*.

Influence of Cellvibrio on the Germination of Spores of Stachybotrys

Since Dobbs and Hinson (1) found that germination of fungal spores was inhibited by natural soil, it was considered that the antagonistic effect of *Cellvibrio* on *Stachybotrys* might also result from an inhibition of spore germination. Petri dishes with 10 ml of liquid medium containing 1% carboxymethylcellulose as carbon source were inoculated with 1 ml of a solution containing 0.5×10^4 *Stachybotrys* spores and 0.1 ml of a suspension of *Cellvibrio* (optical density = 0.4 at 550 mμ) and incubated at 30° C. Inhibition of mycelial growth was observed when compared to controls without bacteria. However, 96% of the spores germinated normally within 8 hours in cultures both with and without *Cellvibrio*. Similar results were obtained with filter paper placed upon agar and inoculated in the same way.

The Fungistatic Effect of Cellvibrio on Stachybotrys

Pieces of sterile filter paper, 1.5 × 1.5 cm, placed on agar medium were inoculated with 0.01 ml of *Stachybotrys* spores. Some of the pieces were simultaneously inoculated with 0.01 ml of *Cellvibrio* culture. The plates were incubated at 30° C. Pieces bearing the mixed populations, which did not show any visible fungal growth, were transferred daily to agar plates containing 100 μg/ml chloramphenicol and incubated for 3 more days. The results are summarized in Table III. The survival of the germinating spores in the

TABLE III

Fungistatic effect of *Cellvibrio* upon *Stachybotrys* on filter paper

Incubation time before transfer to medium containing chloramphenicol (hours)	Number of pieces of filter paper placed on agar	Number of *Stachybotrys* colonies	Colonies*/pieces of filter paper
0	50	248	4.96
8	57	283	4.96
24	52	250	4.80
48	50	219	4.38
96	38	155	4.08
Stachybotrys alone	13	64	4.92

*Average number of fungal colonies per single piece of filter paper.

presence of *Cellvibrio* during 4 days indicated clearly that the effect of *Cellvibrio* upon the vegetative growth of *Stachybotrys* is mainly fungistatic. Statistical analysis of the data showed that there was no significant decrease in number of colonies per piece.

The Effect of Glucose on the Antagonistic Action

Duplicates of Roux bottles containing 100 ml of liquid medium, prepared with various concentrations of glucose, were inoculated either with *Stachybotrys* spores (2.5×10^3/ml final concentration) or cells of *Cellvibrio* (0.5 ml, optical density 0.4 at 550 mμ) or both. After an incubation period of 5 days at 30° C the fungal mats were collected by centrifugation, washed twice with distilled water, dried overnight at 100° C, and weighed. Bacterial growth was determined by measuring the optical density. The results are summarized in Fig. 1. It may be seen that fungal growth was markedly inhibited by *Cellvibrio* only at the low concentrations of glucose, the levels at which bacterial growth responded most noticeably to additional carbohydrate. At higher concentrations of glucose, bacterial response was small and fungal inhibition decreased sharply, until at 2.0 mg/ml little antagonistic effect could be observed.

The quantities of reducing sugar, which remained in the medium after the growth of *Cellvibrio* alone were also determined. It was found that in media containing up to 1.0 mg/ml of glucose (initial concentration), no reducing sugar was left by the bacteria after 5 days of incubation; at higher initial concentrations of glucose, reducing sugar was still present after 5 days

The Effect of Carboxymethylcellulose on the Antagonistic Action of Cellvibrio

When the experiment described above was carried out with carboxymethylcellulose (1%) as the sole carbon source, 12.6 mg mycelium dry weight/100

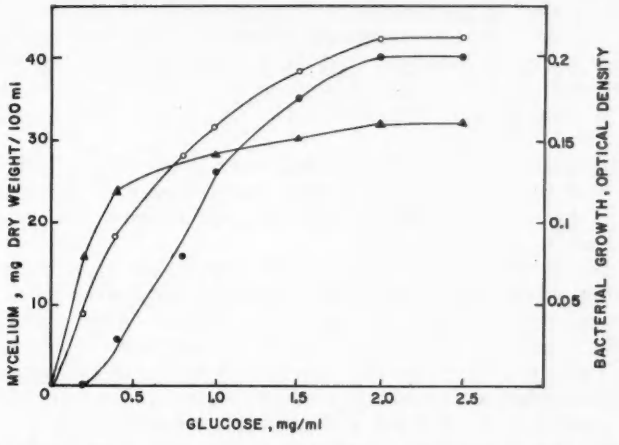

FIG. 1. Effect of various concentrations of glucose on the antagonistic activity of *Cellvibrio* on *Stachybotrys*. ○ *Stachybotrys*. ▲ *Cellvibrio*. ● *Stachybotrys* in the presence of *Cellvibrio*.

ml medium were found when *Stachybotrys* was grown alone, while in the presence of *Cellvibrio* only 3.1 mg/100 ml were found. No reducing sugar was detected.

Discussion

Fungal development from soil crumbs was significantly inhibited when filter paper was used as the sole carbon source. When *Stachybotrys* and *Cellvibrio* strains isolated from soil were inoculated together on filter paper, only *Cellvibrio* developed. As antagonistic effects occurring in nature between microorganisms may be caused by the excretion by one organism of substances toxic to another, such a possibility was considered. There is good evidence, however, that no such substance was formed since growth of *Stachybotrys* could be initiated even after the fungus had been exposed to *Cellvibrio* for some days in the same medium. In addition, no inhibition of the growth of *Stachybotrys* by *Cellvibrio* in a liquid medium was observed when the glucose concentration was sufficient to support growth of both organisms. Apparently, the suppression results from a competition for degradation products of cellulose rather than for cellulose itself, since this competition could also be demonstrated when glucose was used as sole carbon source. The finding that no reducing sugar could be detected whenever antagonism was evident supported the conclusion that a competition for available carbohydrates rather than for cellulose was responsible for the inhibition.

In addition to the production of antibiotics and toxic substances, competition between microorganisms for nutrients is probably also playing a role in microbial interactions in the soil (5, 12). Moreover, available nutrients in the soil are at a much lower level than in ordinary culture media used for antibiotic production (2). Competition for nutrients, however, is not the sole mechanism responsible for inhibition of fungal growth, other mechanisms such as inhibition of germination of fungal spores (1) and lysis of fungal mycelium (4) by unknown substances must be taken into account. Competition for nutrients, however, may be a secondary cause for the lysis of fungal mycelium in soil, as autolysis of mycelium is well known to be much stronger in poor than in rich media (15), i.e., in the absence of available nutrients.

An antagonistic effect of *Bacillus macerans* upon various soil fungi was also observed by Park (8), who studied the behavior of soil fungi in the presence of bacterial antagonists. However, no attempt was made to find out the nature of this antagonism.

The type of cellulose used as substrate seems also to be of importance since inhibition of fungal growth was observed only when filter paper or carboxymethylcellulose was used as carbon source. When cellophane was used as the substrate, fungi grew normally on agar even in the presence of bacteria. This can be explained by the limited ability of the bacteria tested to decompose cellophane on an agar medium. Native and regenerated cellulose differ in a number of chemical and physical properties such as associated substances, crystal structure, properties of the crystalline material, degree of swelling, etc. (6). These differences possibly account for the observed differences in microbial growth when either cellophane or filter paper was

used as a sole carbon source. In contrast to fungi, bacteria lack the ability to penetrate actively into solid substrates (9). In the case of cellophane, fungi grow into this substrate whereas bacteria remain confined to the surface. On filter paper, on the other hand, the competition is apparently on the surface of the microscopic fibrils which are covered with a film of water, and the bacteria apparently dominate in this water film. It may be suggested that bacteria can (by using this water film as a means of transportation), reach the fungal mycelium whenever it grows and compete with it for degradation products of the cellulose.

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EFFECT OF SHORT-CHAIN FATTY ACIDS ON RESPIRATION OF WHEAT STEM RUST UREDOSPORES[1]

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Abstract

Fatty acids added to a suspension of wheat stem rust uredospores (*Puccinia graminis* var. *tritici*, race 15B) stimulate respiration. When compared on the basis of equal molar concentration, the utilization of oxygen by spores from wheat grown in the field or in the greenhouse is stimulated by short-chain fatty acids as follows: acetate < propionate < butyrate < valerate. The order for butyrate and valerate is reversed with spores from plants grown under artificial light. Radiotracer experiments indicate that the amount of respired carbon dioxide derived from the carboxyl carbon of the fatty acid added increases markedly with increase in chain length. The addition of exogenous acetate stimulated conversion of spore carbon to carbon dioxide, whereas valerate replaces, in part, spore material as a source of respiratory carbon. Valerate-1-C^{14} is almost 4 times as effective as acetate-1-C^{14} for labelling spore material, but the ratio of carbon-14 respired to that incorporated is 2.6 for valerate-1-C^{14} as compared with 1.5 for acetate-1-C^{14}.

Introduction

The central role of fat metabolism in respiration of wheat stem rust uredospores (*Puccinia graminis* var. *tritici*, race 15B) has recently been recognized (1, 3, 4, 5). Studies in this laboratory showed that externally supplied fatty acids stimulate the respiration of the spores and that there is a concomitant utilization of the fatty acid for carbon dioxide production, the magnitude of the effect being characteristic of the fatty acid supplied (2). A more detailed examination of fatty acid utilization by the spores is required to understand adequately the significance of these substances in the respiration and germination of spores. Elucidation of such processes may eventually help to resolve uncertainties about the special metabolic characteristics of obligate parasitism or of the phenomenon of self-inhibition of spore germination.

The present work was undertaken to extend the earlier observations on the effects of exogenous short-chain fatty acids on respiration by rust spores. In addition, it was proposed to examine the incorporation of carbon into spore constituents by the use of carbon-14 labelled fatty acids.

Experimental Methods

Wheat stem rust uredospores (race 15B) grown on Stewart wheat were used for all experiments. Host plants were grown in the field, in a greenhouse, or in a growth chamber under artificial light of 1500 ft-c for 18 hours each day. Spores were collected with a vacuum collector and stored at 4° C until used (generally within 24 hours of collection).

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Standard manometric techniques with a Warburg constant volume respirometer were used (2). The main compartment of the Warburg vessel contained the desired concentration of substrate in 3 ml of *M*/30 phosphate buffer which was adjusted to pH 6.2 before use. The rust spores (7.5 mg) were floated on the buffer surface. All measurements were made at 30° C and were done in triplicate.

Carbon-14 was determined by combustion of samples to carbon dioxide which was then counted with a vibrating reed electrometer (Dynacon, Nuclear Chicago).

Experimental Results and Discussion

The respiration rate of rust spores floating on buffer solutions has been shown to vary markedly with the age of the spores (2). Similar observations were made preliminary to work reported herein and it was also noted that the respiration rate of fresh spores depended upon the conditions under which the host plant and spores were grown. However, when a single sample of fresh spores was used with carefully controlled conditions satisfactory replicates were obtained and a consistent pattern of results was observed. The effect of externally supplied fatty acids on the respiration could thus be discerned and satisfactory data on the utilization of these by the spores was obtained.

Table I presents results compiled from numerous experiments widely separated in time and employing different spore samples. The results with spores from plants grown outdoors, in a growth chamber, and in the greenhouse were tabulated separately and show that each responded in a characteristic way. Although three of the values given were obtained with a single determination (each of three satisfactory replicates), the others are averages of two, three, or more determinations. The variability of the data noted indicates that trends observed are significant. Oxygen consumption increased with increase of fatty acid chain length with a single exception. Spores obtained from wheat in the growth chamber consumed less oxygen on valerate than on butyrate. These results agree qualitatively with those of Farkas and Ledingham (2), who found increases of 30%, 45%, 60%, and 49% over basal respiration upon the addition of 0.02 *M* acetate, propionate,

TABLE I

Effect of fatty acids upon rate of oxygen consumption

Substrate added	Increase in oxygen consumption (μl/3-hour interval)*			
	Field**	Growth chamber†	Greenhouse†	Average
Acetate (5.0 m*M*)	13‡	24	33	23
Propionate (5.0 m*M*)	33	35	41	36
Butyrate (5.0 m*M*)	89‡	55	63‡	69
Valerate (5.0 m*M*)	115	51	77	81

*The mean oxygen consumptions in the absence of fatty acid were as follows: Field 84 μl, growth chamber 137 μl, greenhouse 119 μl. The averages of the difference of individual values from the mean were 9 μl, 7 μl, and 10 μl respectively.

†Growth conditions for host plants from which spores were collected.

‡Single determinations. All other values are averages of two or more values. Analysis of variance on data tabulated showed that differences between acids were significant to the 5% level whereas difference between sources of spores were not. The necessary difference between acids for significance to 5% level is 22.5 μl while necessary difference for sources of spores is 25.9 μl.

butyrate, and valerate respectively. Similar results were also obtained by Frear (6), who found that oxygen uptake of germinating flax rust uredospores was increased by 12%, 9%, 21%, and 31% by 0.02 *M* solutions of acetate, propionate, butyrate, and valerate respectively.

Data on the conversion of the carboxyl carbons of the fatty acids to carbon dioxide are condensed in Table II. Spores from several sources were used and all available data were included. Differences between results with different substrates are considerably greater than the deviations recorded. Both the amount of carbon-14 converted to carbon dioxide and the fraction of respired carbon dioxide derived from the carboxyl group of added substrate increased with chain length of fatty acid used, the values with valerate being especially high in comparison to the others.

TABLE II

Utilization of carbon-14 labelled fatty acids*

Substrate	C^{14} converted to CO_2 (% total added)		% of respired CO_2 from COOH of substrate		Stimulation† (% total CO_2)	
Acetate-1-C^{14} (5.0 m*M*)	1.2	(.3)	4.1	(.3)	20	(1.5)
Propionate-1-C^{14} (5.0 m*M*)	2.0	(.1)	7.5	(2.2)		
Butyrate-1-C^{14} (5.0 m*M*)	3.6	(.1)	10.5	(.5)		
Valerate-1-C^{14} (5.0 m*M*)	9.9	(.8)	28	(2.5)	35‡	(8)

*Figures shown in parentheses are averages of the difference between individual values and the mean values.
†The increase in carbon dioxide evolution in the presence of substrate (Stimulation) is expressed as percentage of total carbon dioxide evolved when substrate was present. This was done to make values directly comparable with those in column 3.
‡One sample of spores from the field had a stimulation of CO_2 evolution of 50% of total CO_2 respired. The average stimulation for the remaining values was 28%.

When acetate was added, about 20% more carbon dioxide was evolved than was observed in the absence of substrate. However, only 4.1% of respired carbon dioxide came from the carboxyl group of acetate, and since the methyl group probably accounted for an even smaller amount* it is evident that the increased carbon dioxide was not derived solely from acetate. Thus not only is some acetate oxidized to carbon dioxide, but its presence stimulates conversion of endogenous carbon to carbon dioxide.

The carboxyl carbon released from valerate accounts for 28% of the total carbon dioxide released in the presence of this substrate, and carbon-2 for about 8%.* The observed stimulation of carbon dioxide evolution (35%, see also second footnote, Table II) in the presence of valerate is thus accounted for by carbons 1 and 2 of valerate itself (36% of total CO_2 evolution). Some conversion of carbons 3, 4, and 5 of the valerate to carbon dioxide was also expected. Valerate carbon has, therefore, partially replaced spore carbon as a source of carbon dioxide. Although the above observation demonstrates another marked difference between the effects of acetate and valerate, the data can not be used to explain this difference.

*The contribution of carbons 2 of acetate and of valerate was assessed in separate experiments with acetate-2-C^{14} and valerate-2-C^{14}. The methyl carbon of acetate accounted for about 40% as much carbon dioxide as did the carboxyl group while carbon-2 of valerate accounted for about 30% as much carbon dioxide as did the carboxyl group of valerate.

TABLE III

Incorporation of carbon-14 into spore constituents

	Acetate-1-C^{14} (5.0 mM)	Valerate-1-C^{14} (5.0 mM)
Carbon 14 respired (% added to medium)	1.5	9.5
Carbon 14 incorporated in spores (% added to medium)	1.0	3.7
Total	2.5	13.2
Ratio $\frac{C^{14}\text{ respired}}{C^{14}\text{ incorporated}}$	1.5	2.6

Values for carboxyl carbon respired and carboxyl carbon incorporated were determined using either acetate or valerate (Table III). The experiments were done as described above using fresh spores and 5 mM solutions of acetate-1-C^{14} and valerate-1-C^{14}. Although the carboxyl carbon both of acetate and of valerate is incorporated into the spores, that of the latter was much more effective. The relation is similar to that noted for the carbon-14 evolved as carbon dioxide, and hence the amount of valerate carboxyl carbon taken from the solution is more than 5 times as great as the amount of acetate carboxyl taken up. The preferential utilization of the larger valerate molecule may be caused by a selective permeability of the spore wall, perhaps reflecting differences in the solubility of these acids in the lipoids known to be components of plasma membrane (7).

The differences between valerate-1-C^{14} and acetate-1-C^{14} in the ratio of carbon 14 respired to carbon 14 incorporated (Table III) again show the difference in the metabolic fate of the carboxyl carbons of these two substances. The results suggest either that valerate is utilized by pathways not involving preliminary release of carbons 1 and 2 as acetyl coenzyme A (β-oxidation) or that free acetate is metabolized in part by mechanisms not involving the formation of acetyl coenzyme A.

Acknowledgment

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GROWTH OF MICROCYSTIS AERUGINOSA KÜTZ. IN A PRECIPITATE-FREE MEDIUM BUFFERED WITH TRIS[1]

JACK MCLACHLAN[2] AND P. R. GORHAM

Abstract

A dilute precipitate-free medium has been developed for the culture of *Microcystis aeruginosa* Kütz. With this medium, maximum growth rates of approximately 1.5 divisions per day have been attained during the first 3 days. Buffering between pH 7.4 and 7.7 with 10 m*M* tris (hydroxymethyl) aminomethane (TRIS) caused no toxicity. TRIS increased the tolerance of the alga towards supraoptimal concentrations of monovalent but not divalent cations. High concentrations of magnesium were toxic, but toxicity disappeared when magnesium: calcium ratios of 33:1 or lower were provided. An increase in nitrogen concentration caused no increase in yield, but a decrease caused a reduction. Doubling or halving the concentration of all other salts had no effect on yields. Growth in this medium was apparently limited by factors other than mineral nutrients. This alga has a rather low tolerance for potassium and requires little, if any, sodium.

Introduction

The blue-green planktonic alga *Microcystis aeruginosa* Kütz. is one of the most notorious bloom-producing species. In addition to the obnoxious character of the bloom, some strains of this alga have been found to be toxic (6, 22, 23). Strains of this alga have been cultured successfully (21, 23, 49). Gerloff *et al.* (15, 20, 21) studied minimal mineral requirements of a strain which grew quite slowly and gave low yields from fairly heavy inocula. Hughes *et al.* (23) and Zehnder and Gorham (49) have reported on the development of media and methods for obtaining high yields of another strain. They noted, however, that in some experiments there was an initial lag phase and in all experiments there were no distinct periods of exponential growth.

The *Microcystis* media that have been developed are poorly buffered with carbonate–bicarbonate plus silicate. When a carbonate–bicarbonate medium is autoclaved, permanent damage may occur due to the increase in pH (11). Also, these media tend to develop precipitates when they are autoclaved (49), and once a salt has precipitated, it may redissolve very slowly (9). Another shortcoming of such media is that it is difficult, if not impossible to reproduce the same kind of precipitate (42). This makes it difficult to obtain agreement between replicates or to reproduce the results of an experiment. As a consequence, Droop (11) maintains that a strict control of pH is essential in synthetic media and that these media must be developed in relation to a limited pH range.

Most fresh-water algae frequent neutral or slightly alkaline waters (7), and it generally is assumed that blue-green algae thrive best under alkaline

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conditions (16, 41). Experimental evidence indicates that optimal growth of several species, including *Microcystis*, occurs in the pH range of 7.0 to 11.0 (1, 20, 21, 49).

Several unique nutritional features are characteristic of blue-green algae. Some species are able to fix molecular nitrogen (16), but *Microcystis* does not possess this potential (48, 49). Another characteristic of some species, including *Microcystis*, is an ostensible sodium requirement (1, 3, 12, 21, 28). Some species or strains require either sodium or potassium whereas others require sodium (1). Zehnder and Gorham (49) considered it was necessary to maintain a suitable sodium/potassium ratio for best growth of *Microcystis*. One blue-green alga has been reported not to require calcium or to require only traces of it (20). *Microcystis* and other species, however, have a demonstrable requirement for this element (2, 16, 21) and Zehnder and Gorham (49) have suggested that a calcium deficiency may have been at least partly responsible for lysis of their cultures.

The objects of this investigation were to develop a well-buffered, precipitate-free medium for more rapid and reproducible growth of *Microcystis aeruginosa* without an initial lag phase and to examine the requirements and tolerances of the alga for various constituents of this medium.

Materials and Methods

The alga used in this investigation was *Microcystis aeruginosa* Kütz. emend. Elenkin strain NRC-1 (6, 23, 49), which is not bacteria-free.

The modified ASP medium (32, 33) of Provasoli *et al.* (42), a precipitate-free artificial sea-water medium, was used as the basis for developing a new medium. Sea-water media have been used successfully for growth of other fresh-water algal species (19).

The alga was grown in 35 ml of medium in 125-ml Erlenmeyer flasks plugged with cotton. Cultures were shaken and illuminated continuously. Illumination was provided by cool-white 40-w fluorescent lamps adjusted to an intensity of 2500 to 3000 meter-candles at the level of the shaker, and the temperature was maintained at 23° C ± 1°. Experimental cultures were inoculated (initial O.D. about 0.01) from 8-day-old stock cultures grown under the same conditions as the experimental cultures.

Turbidimetric Growth Determination

Growth was measured turbidimetrically with a Beckman model B spectrophotometer at a wavelength of 750 mμ and a light path of 1 centimeter. Since the chlorophyll content of algae varies widely with the culture conditions (35, 37), Gerloff *et al.* (21) and Miller and Fogg (34) rejected turbidimetry. However, under certain conditions, optical density has been used as a reliable measure of algal growth (25, 28, 36). At 750 mμ there is little or no absorbance by the pigments of *Microcystis*, so the difficulty of pigment variations is obviated. (Optical densities also could have been read in the 300 mμ range, but light scatter is greater at the shorter wavelengths.) Approximately 75 dry-weight determinations of various amounts of growth were made concomitantly with optical density determinations. Known volumes of cell suspensions were passed through membrane filters (Millipore RA), washed

with distilled water and dried to a constant weight. As the tare weight of these filters is quite low, small quantities of the alga could be weighed with reasonable accuracy. The factor "430" was found to be the coefficient of linear regression of dry weight (as μg/ml) on optical density obtained by the method of least squares for absorbances up to 1.0.* In some experiments, therefore, growth is reported in terms of dry weight, calculated from the optical density determinations. All treatments were conducted in triplicate and the results averaged. All experiments were terminated after 8 days unless otherwise noted.

Results

1. Development of the Precipitate-free Medium

A comparison of the composition of the modified ASP medium of Provasoli *et al.* (42) with that of the dilute, precipitate-free medium, called ASM, developed from it is presented in Table I.

TABLE I

A comparison of the composition in micromoles per liter of modified ASP and ASM media*

	Modified ASP	ASM
NaCl	410×10^3	—
$MgSO_4$	24×10^3	200
$MgCl_2$	20×10^3	200
$CaCl_2$	10×10^3	100
KNO_3	1000	—
$NaNO_3$	—	1000
K_2HPO_4	100	100
Na_2SiO_3	100	—
Na_2EDTA†	30	20
$FeCl_3$	1.5	2
H_3BO_3	185	10
$MnCl_2$	7	7
$ZnCl_2$	0.8	0.8
$CoCl_2$	0.02	0.02
$CuCl_2$	0.0002	0.0002

*Media prepared with deionized distilled water.
†Disodium ethylenediaminetetraacetate.

In developing the ASM medium, the concentrations of nitrogen, phosphorus, micronutrients, and disodium ethylenediaminetetraacetate (Na_2EDTA) were first kept the same as in ASP medium, while the other salts were diluted from 0.1 to 1.0 times their concentration in ASP medium. Potassium nitrate was replaced by sodium nitrate, as Zehnder and Gorham (49) obtained poor growth in their medium when nitrogen was offered as potassium instead of sodium nitrate. Sodium silicate was omitted as it has been found necessary only for growth of diatoms. A limited amount of growth occurred only in the 0.1 times dilution.

When the boron concentration was reduced from 185 to 10 μM (but retained as an essential micronutrient (14, 31) and the ratio of chelator to micronutrients

*The statistical analysis was carried out by Mr. N. T. Gridgeman of the Division of Applied Biology.

was reduced from 3:1 to 2:1, growth of *Microcystis* was still poor, but when sodium chloride also was omitted good growth of the alga was obtained. The concentration of magnesium and calcium was further reduced 10-fold without decreasing growth of the alga.

Growth was reduced if potassium nitrate was substituted for sodium nitrate. Since Zehnder and Gorham (49) had suggested that a Na/K ratio of less than 1.0 inhibited growth, the possibility that reduced growth might have been caused by too low a Na/K ratio or by sodium deficiency was investigated. Using potassium nitrate as the source of nitrogen, various concentrations of sodium chloride were introduced into the medium to a maximum Na/K ratio of 1.0. The best growth occurred in the controls with sodium nitrate, so it was tentatively concluded that the alga has a low tolerance for potassium.

The pH of ASM medium was adjusted to 7.4 to 7.6 before autoclaving, but after autoclaving, the pH decreased to about 6.8. With substantial growth in this medium the pH sometimes rose to values exceeding 10.

2. pH Control

Tris (hydroxymethyl) aminomethane (TRIS) is a buffer that is extensively employed in marine algal media for the pH range 7.0 to 8.5. Zehnder and Gorham (49) obtained no growth of *Microcystis* at pH 8.4 using 10 m*M* TRIS. Growth occurred only when the concentration of TRIS was reduced to the point where it had little or no buffering capacity. Since it has been found with other algae that TRIS may exaggerate the potassium requirement by antagonizing its uptake (30, 39, 42), Zehnder and Gorham suggested that the TRIS toxicity they observed may have been due indirectly to potassium deficiency.

The effects of various concentrations of TRIS from 0 to 10 m*M* were tested

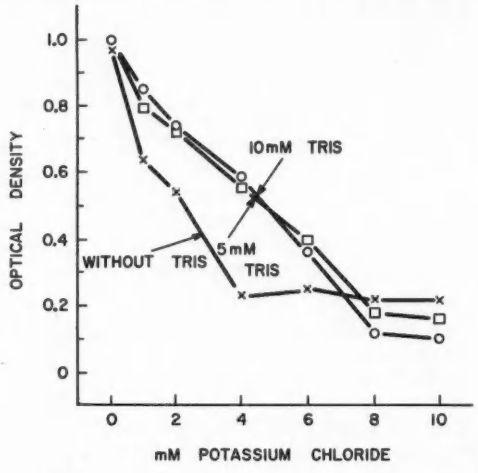

FIG. 1. The effect of various concentrations of potassium chloride on growth of the alga without and with 5 m*M* and 10 m*M* TRIS. Harvest was made after 8 days of growth.

in ASM medium adjusted to pH 7.4 before autoclaving. As ASM medium has a low potassium content (0.2 m*M*), one m*M* potassium chloride was added to prevent a possible TRIS-mediated potassium deficiency. After autoclaving, the pH was 7.3 to 7.4 with the various concentrations of TRIS. Growth was equally good in all cultures. The final pH with 0 and 2 m*M* TRIS was 7.9 and 8.4 respectively; with 4 to 10 m*M* TRIS it varied from 7.9 to 7.6 Buffering was therefore adequate at TRIS concentrations of 4 to 10 m*M*, and there was no suggestion of toxicity.

The suspected low tolerance of the alga for potassium was confirmed by an experiment in which 0 to 10 m*M* potassium chloride was added to ASM medium containing 0, 5, or 10 m*M* TRIS (Fig. 1). The tolerance for potassium was increased, however, by the addition of 5 or 10 m*M* TRIS.

3. Rate of Growth in ASM Medium with 10 m*M TRIS*

The initial pH of the ASM medium containing 10 m*M* TRIS was adjusted to 7.4, and harvests were made daily for a period of 12 days. Exponential growth was maintained for approximately 3 days with no indication of a lag phase (Fig. 2). During the period of exponential growth, the cell density increased about 1.5 times per day. The daily increments in cell density declined after the third day, so that the total additional increase during the next 9 days was only about 1.5 times.

Since ASM medium with 10 m*M* TRIS supported good growth of the alga without an apparent lag phase, this concentration of TRIS was adopted

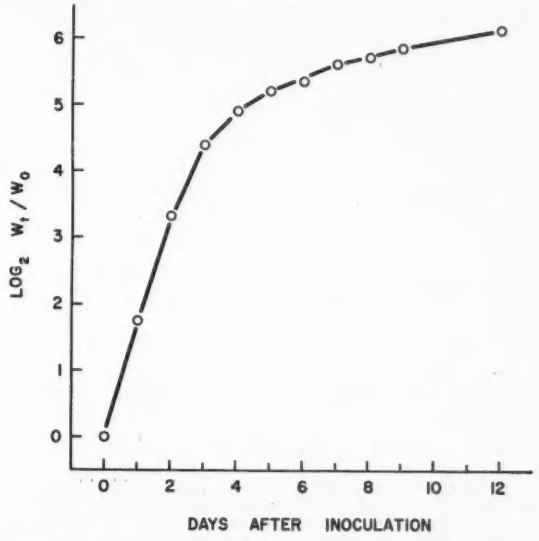

FIG. 2. The rate of growth of *Microcystis* in ASM medium with TRIS. The values for dry weight have been calculated from optical density readings. W_0 is the calculated value at the time of inoculation, and W_t the value at the time of harvest.

for use in all subsequent experiments. The initial pH was always adjusted to 7.4. The maximum optical density obtained with this medium under the culture conditions described was around 1.00 and will be referred to as "normal growth".

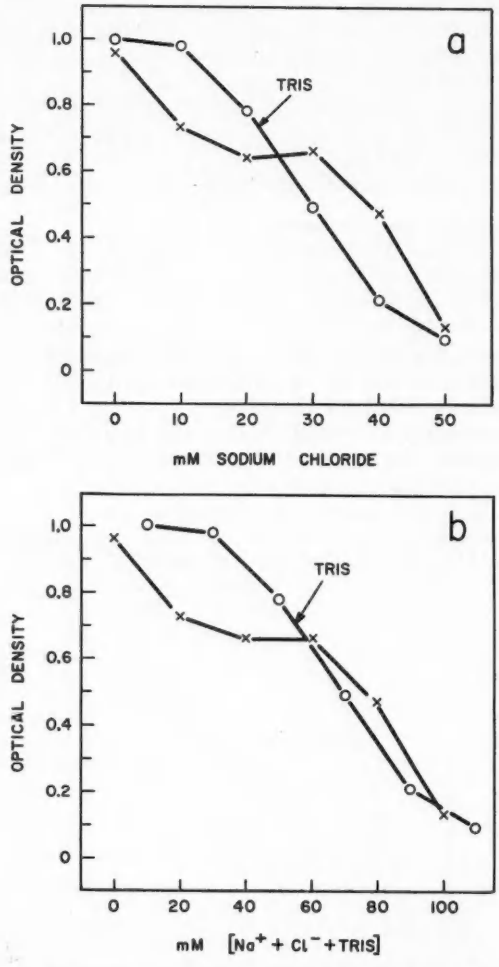

FIG. 3*a*. The effect of various concentrations of sodium chloride on the growth of the alga with and without TRIS. Harvest was made after 8 days of growth.

FIG. 3*b*. The effects of various concentrations of sodium chloride on growth with and without TRIS. Data are the same as in Fig. 3*a*, but the ionic concentrations of sodium and chloride and the concentration of TRIS are added together.

4. Monovalent Cations vs. TRIS

Since TRIS lessened the inhibition of growth caused by excessive potassium, its effects with other monovalent cations were examined. Concentrations of sodium chloride from 0 to 50 m*M* were added to ASM medium with and without TRIS. With 10 and 20 m*M* sodium chloride, growth of the alga was less inhibited with TRIS, but with 30 and 40 m*M* sodium chloride, it was less inhibited without TRIS (Fig. 3*a*). The reason for this apparent reversal of effect on inhibition at high sodium concentrations became clearer when results were compared on the basis of the total ionic concentration of sodium chloride plus TRIS (Fig. 3*b*). At concentrations of 60 m*M* or less, TRIS alleviated sodium toxicity in much the same way that it alleviated potassium toxicity (Fig. 1). At concentrations greater than 60 m*M*, the reduction in growth in both media was approximately the same. The high osmotic pressure or a combination of high osmotic pressure and excessive sodium apparently limited growth.

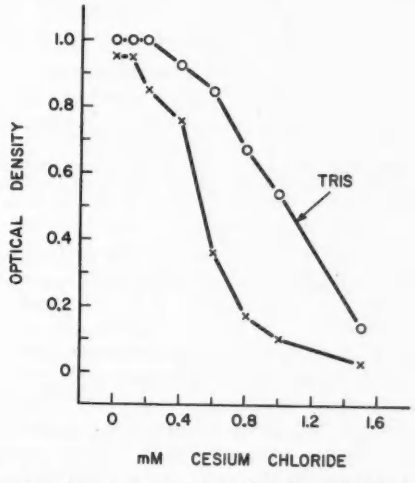

FIG. 4. The effect of various concentrations of cesium chloride on growth of the alga with and without TRIS. Harvest was made after 8 days of growth.

The growth of the alga was less inhibited by cesium in the presence than in the absence of TRIS (Fig. 4). Growth in both media was almost completely suppressed with 1.5 m*M* cesium. With rubidium, normal growth without TRIS occurred at 0.2 m*M*; with TRIS at 0.4 m*M*. No growth occurred in either medium at a rubidium concentration exceeding 2.0 m*M*. In both media the concentration of lithium exceeded 0.8 m*M* before growth inhibition occurred. At higher concentrations, the toxic effects of lithium were less pronounced with TRIS, but growth was almost completely suppressed in both media by 1.5 m*M* lithium.

In the presence of 0.6 m*M* cesium, the rates of growth both with and without TRIS were essentially the same for the first 2 days (Fig. 5). How-

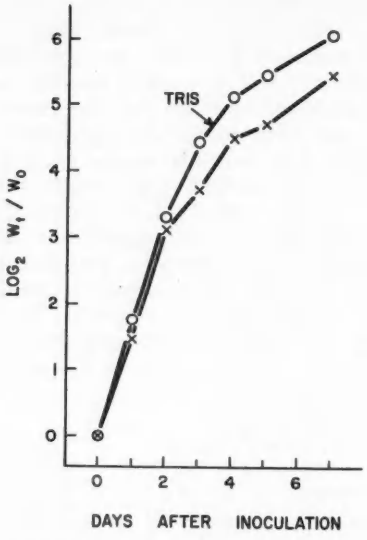

FIG. 5. The rate of growth of the alga in ASM medium containing 0.6 m*M* cesium chloride with and without TRIS. The values for dry weight have been calculated from optical density readings.

ever, after the second day, the rate of growth was more rapid with TRIS than without. Similar results were obtained with potassium and rubidium.

5. Divalent cations vs. TRIS

The concentration of magnesium chloride was varied from 0 to 20 m*M* in the presence and absence of TRIS. Magnesium sulphate was replaced by 0.2 m*M* sodium sulphate as the source of sulphur. The magnesium/calcium ratio varied from 0:1 to 200:1. Toxic effects of excessive magnesium were not mitigated by TRIS. Cultures without added magnesium grew approximately 50% less than the controls. With both media, normal growth occurred at a magnesium concentration of 0.4 (control) and 1.0 m*M*; at 5.0 m*M* growth was reduced, and no growth occurred from 10 to 20 m*M*. Even at the highest magnesium concentration the osmotic pressure was not great enough (based on the sodium chloride experiment) to suppress growth completely.

The concentration of calcium chloride was varied from 0 to 5.0 m*M* in media with and without TRIS. The magnesium/calcium ratio ranged from 4:0 to 4:50 (or 0.08:1). At calcium concentrations greater than 3.0 m*M*, a slight precipitate developed in the ASM medium. No precipitate was noted in the medium with TRIS at any of the calcium concentrations. No growth occurred in those cultures from which calcium had been omitted. In all other cultures, both with and without TRIS, growth was approximately the same. Therefore, both the calcium concentration and the magnesium/calcium ratio occurring in the ASM medium are favorable for growth of the alga.

In media with and without TRIS, concentrations of magnesium chloride were varied from 0.4 to 20 m*M* and calcium chloride from 0.1 to 5.0 m*M* in such a way as to maintain the magnesium/calcium ratio at 4:1. Sulphate was supplied as sodium sulphate. Growth was approximately the same in all cultures with no indications of inhibition. The magnesium toxicity noted initially at concentrations of 5.0 m*M* and higher was overcome by maintaining a favorable magnesium/calcium ratio.

The magnesium/calcium ratio was varied from 0.06:1 to 200:1 in the medium with TRIS. No growth occurred at ratios of 100:1 or greater, very poor growth occurred at 66:1, but normal growth occurred from approximately 33:1 to 0.06:1. As demonstrated in the previous experiment, the magnesium/calcium ratio is more important in promoting good growth than the actual concentrations of these two elements as long as they do not fall below critical levels. The highest permissible magnesium/calcium ratio at which normal growth can occur lies somewhere between 33:1 and 50:1.

6. Micronutrients

Concentrations of ferric chloride were varied from 0 to 10 μM in ASM medium with TRIS. At the highest iron concentration, the ratio between micronutrients and chelator was 1:1. In those cultures to which iron was not added it was estimated that approximately 60 $\mu\mu M$ were introduced by carry-over with the inoculum plus contamination from the other salts. Only about a 10% reduction in growth occurred in the cultures without added iron. All other cultures reached the same optical density after 8 days of growth. Therefore, it was concluded that the concentration of iron in the ASM medium is in the optimal range for the culture conditions employed.

The concentration of micronutrients, including iron and chelator, in ASM medium was varied from 0 to 8 times normal in the presence of TRIS. There was a slight reduction in growth ($<10\%$) in cultures where these compounds were omitted, but normal growth occurred in all other cultures. Since a similar reduction in growth occurred when iron alone was omitted it was concluded that the concentration of micronutrients and chelator normally supplied in the ASM medium is neither limiting nor inhibitory to growth of the alga.

7. Total Salts and Major Salts

The concentration of all salts in the ASM medium was varied from 1/16 to 4 times normal in the presence of TRIS. Normal growth occurred at concentrations of 1 to 4 times normal. At concentrations below normal, growth was reduced.

The effect of varying the concentrations of each of the major salts from 0.5 to 2 times normal was tested in the presence of TRIS. Sodium nitrate was the only salt that limited growth at 0.5 times normal; the decrease was about 70% of normal. This result is similar to that obtained by Gerloff *et al.* (21) with their strain of *Microcystis*. There was no increase in growth in any of the cultures containing twice the normal concentration of any one salt. An N/P ratio of at least 20:1 is not too high for normal growth.

An attempt was made to increase the final yield of the alga in ASM medium with TRIS by increasing the concentration of nitrogen to 3 times normal and

the concentration of phosphorus to 2 times normal. To offset the possibility that these cultures might become carbon limited, a parallel series containing 1 m*M* sodium carbonate was included in the experiment. After 14 days of growth, the control cultures had an optical density of 1.25. Cultures containing increased nitrogen and phosphorus (both with and without carbonate) had an optical density of approximately half that of the controls. Something other than the ASM nutrients apparently limits maximum cell density.

8. Molybdenum Concentration

As the ASM medium does not contain molybdenum, a test was made to see if a deficiency of this element might be limiting nitrate reduction (cf. 4, 18) and hence, growth. Ammonium molybdate was added to ASM medium with TRIS at various concentrations from 7 to 21 μM. For each molybdenum concentration, one set of cultures was prepared with the normal concentration of sodium nitrate and one set with twice the normal nitrate concentration. The cultures were incubated for 14 days. Increased nitrogen and the addition of molybdenum caused no increase in cell density, therefore there was no apparent deficiency of molybdenum.

9. Omission of Sodium and Chloride

Growth was tested in a modified medium in which sodium and chloride were omitted. (Probably no more than 40 μM sodium was present from other sources.) This medium contained 2.00 m*M* magnesium sulphate and 0.5 m*M* calcium nitrate. The calcium and magnesium concentrations were increased by a factor of 5, but the 4:1 ratio was still maintained. The other salts, plus TRIS, were added at the same concentration as in ASM medium. *Microcystis* grew well in this medium and was carried through a large number of transfers over a period of many months. The rate of growth and final cell density were similar to those obtained with ASM medium (Fig. 2).

Discussion

ASM medium with 10 m*M* TRIS provides a well-buffered, precipitate-free medium in which all constituents are present in concentrations that appear to be neither limiting nor in excess for good growth of *Microcystis aeruginosa* strain NRC-1. This medium eliminates the initial lag phase and lack of a period of exponential growth noted by Zehnder and Gorham (49). Final yields were not as great as those obtained by Zehnder and Gorham (49), but exceeded those obtained by Gerloff *et al.* (21). Final yields apparently were limited by factors other than mineral nutrients. Although it has been stated (25, 26) that the presence of bacterial contaminants has little or no effect on growth responses of algae in relation to mineral nutrition and physical factors, this point has not been adequately established. The rate of growth was as fast or faster than that of most other blue-green algae (cf. 29, 49) with the exception of *Anacystis nidulans* (28). Replicate variability was small, and consistent and reproducible results were obtained between experiments, presumably because there was no precipitation in the medium.

ASM medium with TRIS does not differ radically from other fresh-water media developed for planktonic forms (cf. 10, 44, 46), except that it is well

buffered. Contrasted with such media as have been developed for other blue-green algae by Kratz and Myers (28) and Allen and Arnon (2), it is quite dilute. However, the osmotic strength is comparable to the media of Gerloff *et al.* (20, 21) and Zehnder and Gorham (49). The magnesium/calcium ratio is high, but these two elements are known to be widely interchangeable (33, 42). The monovalent/divalent ratio has been considered an important factor influencing the growth of algae (cf. 17, 34, 43), but *Microcystis* grew well with ratios ranging from 0.08 to 26.0. Limiting ratios were not determined, but in fresh-water media the magnitude of this ratio depends chiefly upon the quantity and form in which nitrogen is provided since it is the major component of most such media.

Gerloff *et al.* (21) showed that their strain of *Microcystis* had a very distinct pH optimum at 10, whereas Zehnder and Gorham (49) found strain NRC-1 had a rather broad optimum range between pH 8 and 11. Strain NRC-1, in ASM medium buffered between pH 7.4 and 7.7, grew at a faster rate than obtained in flasks in either of the previous investigations. Both of the previous studies reported a growth lag when silicate and carbonate were omitted from the media; this was attributed to a low initial pH. Neither of these salts is present in ASM medium. The rate of growth in ASM was essentially the same as in buffered ASM even though the initial pH of ASM was lower. It would seem that the relation of pH to the composition of the medium is more important for the growth of *Microcystis* than pH per se.

Zehnder and Gorham (49) found that at pH 8.4, 10 m*M* TRIS was toxic to strain NRC-1. However in ASM medium buffered with 10 m*M* TRIS at pH 7.4, growth was not inhibited. The effects of TRIS seem to vary from one organism to another, and it may be that both pH and composition of the medium influence the response to TRIS. Further studies of the effects of TRIS on algal growth are needed.

The presence of TRS mitigated the inhibitory effects of excess potassium or other monovalent cations. Since TRIS neither binds cations (30) nor serves as a nitrogen source (8), the most probable mechanism of action is that TRIS antagonizes uptake (30, 39, 42). However, a study with soybean roots has shown that 200 m*M* TRIS has no effect upon potassium accumulation (5). Hydrogen-ion concentration may be an important factor. Some studies have suggested that cation accumulation is enhanced by suitable changes in pH (5, 13, 24, 38, 45, 47). However, the mechanism regulating penetration of ions is not adequately understood. Most accumulation studies have been concerned with absorption from single salt solutions, and it is well known that the presence of several ions complicates the situation (e.g. 5, 13, 24, 27). Studies with radioisotopes may provide a plausible explanation of the effect of TRIS on monovalent cation tolerance.

Although the toxic effects of excess magnesium or calcium were not mitigated by TRIS, there was an interaction between these two divalent cations. The toxic effects of excess magnesium disappeared when the magnesium/calcium ratio was 33:1 or lower. Since good growth occurred with quite widely varying concentrations and ratios of these two cations, it is probable that more than uptake antagonism is involved in their interaction.

The order of toxicity of the monovalent cations for growth of *Microcystis* was Na < K < Li < Cs ≈ Rb. This order was the same regardless of the increased tolerance caused by the presence of TRIS. Pirson (40) mentions that with single salt solutions the order of toxicity of alkali chlorides for plants is Na < K < Rb < Cs < Li, although in some cases sodium is more toxic than potassium. Zehnder and Gorham (49) observed that *Microcystis* was quite sensitive to potassium concentration, and this has been confirmed. Provasoli and Pintner (44) point out that planktonic organisms may be inhibited by as little as 30 mg% of sodium chloride; *Microcystis* was found to tolerate approximately twice this concentration. Allen and Arnon (3) found their culture of *Anabaena* could tolerate at least 10 p.p.m. of cesium, or lithium, and *Microcystis* tolerated approximately the same concentrations. In general, the tolerance of *Microcystis* to monovalent cations seems to be similar to the tolerance of these other organisms.

Microcystis attained maximum growth in buffered ASM medium at a N/P ratio of 20:1. This is in general agreement with the findings of others (20, 21, 32, 34, 49). Arnon *et al.* (4) and others (18) have shown that molybdenum is necessary for nitrate reduction. However, Pintner and Provasoli (39) did not get improved growth of *Phormidium* by concomitantly increasing the nitrogen and molybdenum concentrations, and growth of *Microcystis* did not increase under similar conditions.

Some blue-green algae, including *Microcystis*, require sodium (1, 3, 12, 16, 21, 28, 41). Allen and Arnon (3) found this requirement to be of major proportions (i.e. 5 p.p.m.) for *Anabaena cylindrica*. It was possible to obtain good growth of strain NRC-1 in a medium containing as little as 40 μM sodium. If this strain requires sodium, it is only in micronutrient quantities.

Zehnder and Gorham (49) indicated that lysis of strain NRC-1 was related to the sodium/calcium balance of their medium. Lysis of strain NRC-1 was never observed in the ASM medium with TRIS. Other strains of *Microcystis*, however, lysed in this medium as well as in the medium of Zehnder and Gorham (unpublished). Consequently, the problem of lysis was not resolved, but only circumvented in the case of strain NRC-1.

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SOME ASPECTS OF PHOSPHATE METABOLISM OF CLAVICEPS PURPUREA (FR.) TUL.

II. RELATIONSHIP BETWEEN NUCLEIC ACIDS SYNTHESIS AND ERGOT ALKALOID PRODUCTION[1]

C. DE WAART[2]

Abstract

A study was made of the distribution of the phosphorus-containing compounds in the phosphate pool of stationary cultures of *Claviceps purpurea* (Fr.) Tul. Exogenous KH_2PO_4 was mainly converted to ribonucleic acid. Conditions favorable to increased nucleic acid synthesis increased the yield of ergot alkaloids. The proportion of nucleic acid and acid-insoluble phosphate fraction appeared to be an important factor influencing the ergot alkaloid production.

Introduction

In a previous communication (11) it was reported that the amount of phosphate which permits ergot alkaloid (EA) synthesis by *Claviceps purpurea* in culture is insufficient for maximum rate of growth of the organism. The EA production started after the distribution of the several complex phosphates in the phosphate pool became momentarily constant. An observed release of polyphosphates and nucleic acids from the mycelium into the medium could not be explained.

It is the purpose of the present paper to correlate certain metabolic factors conditioning the accumulation and disappearance of nucleic acids and polyphosphates during the cultivation of *Claviceps purpurea*. In order to achieve this, the following phenomena are described: (*a*) the differences in the phosphate distribution in the phosphate pool between EA-producing cultures and restricted EA-producing cultures during the growth period of the fungus with the same initial amounts of KH_2PO_4; (*b*) the influence of inhibiting and stimulating agents on the EA yields, and on the phosphate pattern. In this part of the work various purine derivatives were used because of a relationship suggested by the large amounts of synthesized ribonucleic acid (RNA) which were detected; and (*c*) the influence of energy-rich pyrophosphate on EA production and on the phosphate pattern.

Materials and Methods

Cultivation and Preparation of the Organism

The organism used throughout this study was *Claviceps purpurea* (PRL 1578). It was grown in stationary culture in 250-ml Erlenmeyer flasks, each containing 40 ml autoclaved basal medium (7), 10 ml filter-sterilized carbohydrate solution enriched with 0.25 μg biotin, and, in some experiments,

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adenosine triphosphate (ATP) or other agents were added. One milliliter of prepared inoculum (8) was used per flask. Compositions of the media have been described elsewhere (7). Commercial galactose (Difco) was used. Purine derivatives and RNA were obtained from Nutritional Biochemicals Corporation. A solution of 2.5 mg RNA in 100 ml 5% trichloroacetic acid (TCA) ($\epsilon = 0.69$) was used as reference standard. The RNA contained 9.6% phosphorus.

Fractionation Procedure and Analyses

Following incubation of the stationary cultures, the mycelia from the different flasks were collected individually by filtration on Buchner funnels. After it was washed with distilled water, the mycelium was homogenized (Potter–Elvehjem homogenizer) in 40 ml 5% TCA at 0–4° C, and analyzed by modifications of previously reported methods (4, 5, 10) (Table I). Orthophosphate was determined colorimetrically according to Sumner (6) in the filtrate and the extract. Complex and total phosphate were determined as orthophosphate in extracted mycelium, in the filtrate, and in the extract, by digesting the sample with sulphuric acid (1) before the orthophosphate assay was carried out.

TABLE I

Sequence of various steps used in analyses

Source	Phosphate analyzed	Method
Filtrate	Ortho	Directly (2, 6)
	Complex	Digestion (1)
	Nucleic acids (RNA)	Ultraviolet (265 mμ)
Mycelium 24 hours cold 5% TCA extract	Ortho or easily hydrolyzable poly	Directly
	Ten minutes 95° C 1 *N* HCl hydrolyzed poly/pyro	Directly
	Total	Digestion
	Nucleic acids (RNA)	Ultraviolet spectrum
One-half hour hot 5% TCA extract (95° C)	Hydrolyzed poly/pyro	Directly
	Total	Digestion
	Nucleic acids (RNA)	Ultraviolet spectrum
Mycelial residue (not acid-extractable)	Total	Digestion

Orthophosphate in TCA extracts and in the filtrate was estimated colorimetrically by direct analysis after it was found that this method gave essentially the same results as when samples were analyzed by quantitative precipitation of the orthophosphate with $CaCl_2$ at pH 8.8 (2).

RNA contents were estimated with a Beckman DK Automatic recording spectrophotometer. Measurements were made at the wavelength of 265 mμ to eliminate the interference at 258 mμ caused by the TCA. Satisfactory ultraviolet estimation of RNA by this procedure was impossible in a few

samples which contained a considerable amount of pigment and unknown quantities of other absorbing materials. Procedure A of Vining and Taber (9) was used routinely to estimate the ergot alkaloid yield. The results were recorded to within 5 mg/liter, because of the range of variation shown by the cultures.

Results and Discussion

Ergot alkaloid-producing cultures differed markedly from those not producing EA in the distribution of phosphates in: (*a*) the amount of TCA-extracted RNA; (*b*) the total phosphate in the mycelium residue, and (*c*) the soluble phosphates (i.e. the sum of the filtrate phosphates plus TCA-extracted phosphates minus TCA-extracted RNA).

Figure 1 shows the differences in the phosphate distribution between EA-producing and non-producing cultures of the same strain and with the same initial concentration of KH_2PO_4 in the medium, but with commercial galactose as a carbon source for the one and purified galactose for the other (7).

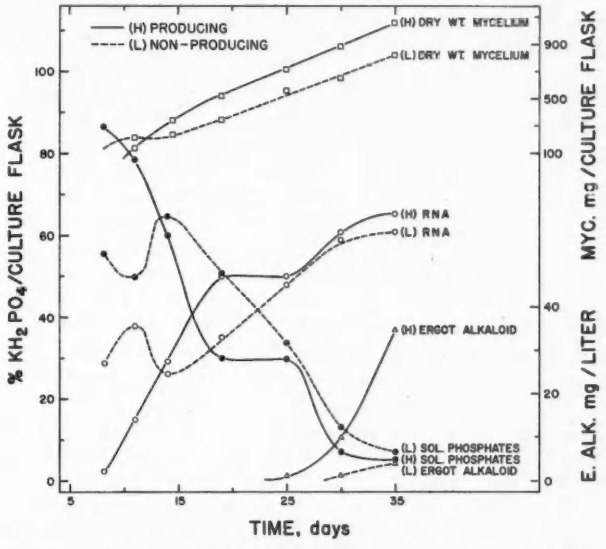

FIG. 1. Difference in phosphate distribution during growth of ergot alkaloid-producing and non-producing cultures. Values are calculated as percentage of phosphate of the initial KH_2PO_4. ○ Ribonucleic acid in cold and hot TCA extract. ● Soluble phosphates, i.e. sum of all the phosphates found in the filtrate and the non-RNA phosphates in the TCA extract.

The "rest period" in the RNA synthesis immediately before the initiation of EA production appears to be typical. Restricted EA production, due to changes in nutritional requirement, showed a release of nucleic acids and other complex phosphates into the filtrate in the period of most active growth. Perhaps this leakage can be explained on the basis that the organism requires

TABLE II

Effects of some purines and a pyrimidine on ergot alkaloid production

Treatment per culture flask		Ergot alkaloids in mg per liter	
		36 days	41 days
Control		45	60
ATP	1.25 mg	45	70
ATP	2.5 mg	75	60
ADP	2.5 mg	10	15
Adenylic acid	2.5 mg	10	25
Uracil	2.5 mg	5	15

TABLE III

Effects of various compounds on phosphates and ergot alkaloid production

Treatment per culture flask		Expressed as percentage PO_4 of initial KH_2PO_4			
		TCA-extracted RNA	Other soluble phosphates	Residue mycelium phosphates	Ergot alkaloid, mg/liter
30 days					
Control		58	17	25	30
ATP	2.5 mg	65	10	25	45
ATP	5 mg	54	31	15	10
ATP*	10 mg	44	44	12	5
36 days					
Control		65	7	28	45
Pyridoxal phosphate		68	3	29	55
Adenosine	2.5 mg	54	22	24	15
Adenosine	1.25 mg	64	8	28	40
Adenine	2.5 mg	55	19	26	20
Adenine	1.25 mg	58	15	27	30

*Ten milligrams ATP represents an amount of KH_2PO_4 that does not change the normal metabolism pattern and EA production.

TABLE IV

Changes in phosphate distribution during 35 days' incubation, expressed as percentage phosphate of initial KH_2PO_4

	Days' incubation				
	8	15	20	26	35
Filtrate orthophosphate	52	14	4	1	1
Mycelium orthophosphate	4	10	9	8	5
Acid-soluble polyphosphate	22	34	18	17	5
Acid-insoluble polyphosphate	16	12	22	24	24
Ribonucleic acid	16	20	48	50	65

a great deal of energy, and little high-energy phosphate could accumulate as polyphosphate or nucleic acid at that time. This release effect is most obvious when commercial galactose is used as a carbon source or when considerably more KH_2PO_4 is used than the optimum of 250 mg/liter. However, when the EA production begins in the restricted culture, a rest period in the synthesis of RNA is also noted. This stationary phase did not affect the mycelial growth of the EA-producing culture, as did the release of phosphate in the non-producing culture. Probably it can be assumed that during the rest period the mycelium is already in such a state that it no longer requires such large amounts of energy for growth and RNA synthesis, presumably enabling "high energy" phosphate to be stored as polyphosphate (Table IV), and (or) to initiate the EA production. Eventually both cultures consumed the various kinds of acid-soluble phosphates by converting them into RNA, and reached finally the same RNA level.

The phosphate pattern described above, however, indicates that the restricted EA production may not be caused by inhibitors, for inhibitors show another picture. The evidence indicates that EA production requires a rather large amount of RNA along with a small amount of other acid-soluble phosphates.

Table IV shows that RNA synthesis is accompanied at first by formation of acid-soluble and -insoluble polyphosphates and later by utilization of acid-soluble polyphosphates. This may show that the two polyphosphate fractions which are separated by TCA extraction have different metabolic activities. The different activities may be due to the difference in protein complexing properties of polyphosphates of different chain lengths (3, 4).

The fact that RNA accumulates from the polyphosphate and (or) the pyrophosphate present strongly suggested using inhibitors and stimulators of this process in further experiments.

To this end, several purine derivatives were selected which, in view of their structure, might affect the RNA synthesis. Among these was the nucleotide ATP which possesses the terminal energy-rich pyrophosphate group which could contribute to both sides of the phosphate balance. The effects of some purines and a pyrimidine on the EA production in a screening experiment are shown in Table II.

An ATP level of 2.5 mg per flask stimulated EA production (Table II). Pyridoxal phosphate also stimulated EA production (Table III). The other purine derivatives slowed down the EA production. Some interesting facts were discovered when experiments were run on the fate of phosphate.

High RNA synthesis, good EA production, and low acid-soluble phosphate level occurred at the same time (Table III). An inhibited EA production showed less RNA and more acid-soluble polyphosphates present. In all cases, the acid-insoluble fraction remained at the same level.

This indicates that RNA formed in this kind of culture strongly depends on the preformed amount of "suitable" low-polymerized metaphosphates and (or) pyrophosphate. Added ATP stimulated EA synthesis, but an abundant supply of ATP acts the opposite way. The high energy bonds conveyed by ATP can be used for immediate metabolic needs (it actually has disappeared

within 6 days from the filtrate), but excess of ATP mobilized the "fixed" phosphate from the mycelium in competition with the synthesis of RNA.

Pyrophosphate probably acts as a factor which limits the synthesis of nucleic acids and also prevents a build-up of the high polymer phosphates. This is contrary to the normal purine inhibition that affects only the synthesis of RNA, leaving the acid-insoluble polyphosphate in the mycelial residue untouched. It leads to the conclusion that ATP inhibits competitively in the phosphate pool while the other purine derivatives act non-competitively. Promoting the RNA synthesis serves to protect and stimulate the EA production and the required enzyme formation involved.

Acknowledgments

The author wishes to thank Dr. W. A. Taber for his stimulating interest and Mr. D. W. Mansfield for technical assistance.

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FACTORS INVOLVED IN THE BACTERIAL DECARBOXYLATION OF SUCCINIC ACID[1]

A. A. YOUSTEN AND E. A. DELWICHE

Abstract

The succinic decarboxylase system of *Veillonella alcalescens* loses activity when aged at 2° C for a few days. Evolution of CO_2 by aged extracts of this organism was greatly stimulated by boiled cell-free extracts of *Propionibacterium pentosaceum*. These boiled extracts could not be replaced by coenzyme A (CoA), adenosine triphosphate (ATP), magnesium ions, biotin, or dimethylbenzimidazolylcobamide coenzyme (DBC).

Introduction

The biological conversion of succinic acid to propionic acid and CO_2 has been intensively investigated in several laboratories. The reaction mechanism is complex. Succinyl-CoA has been identified as an intermediate. Succinyl- and propionyl-CoA transferase activities have been shown in extracts of *Micrococcus lactilyticus* (9), *Propionibacterium pentosaceum* (2), and *Veillonella alcalescens* (2). Evidence provided by exchange reactions in which more propionate-2-C^{14} than $C^{14}O_2$ was incorporated into succinate led Phares *et al.* (5) to postulate that a complexed "C_1" fragment was the initial product of succinate decomposition and that CO_2 was subsequently produced from this intermediate. Methylmalonyl-CoA is an intermediate in the conversion of propionyl-CoA to succinyl-CoA by animal tissue (3), and its role in the reverse reaction has been demonstrated in *Propionibacterium shermanii* (8), *P. pentosaceum* (4), and *V. alcalescens* (4).

Recent evidence (4, 6, 8) has established a coenzyme function for biotin in the decarboxylation process, and is in verification of the earlier observation (1) that biotin-deficient cells of *P. pentosaceum* can be greatly stimulated in the production of CO_2 from succinate by the addition of biotin. Evidence for a vitamin B_{12} coenzyme functioning in the isomerization of succinyl-CoA to methylmalonyl-CoA in *P. shermanii* has been obtained (6, 7).

Cell-free preparations of *P. pentosaceum*, unlike suspensions of intact cells, produce only insignificant amounts of CO_2 in the conversion of succinyl-CoA to propionyl-CoA. Cell-free extracts of *V. alcalescens*, however, are very active in the production of *both* CO_2 and propionate from succinate. This property is lost upon aging of the extracts in the dark for a few days at 0–2° C, and neither propionate nor CO_2 is then produced. When preparations of *P. pentosaceum* and aged extracts of *V. alcalescens* are combined, CO_2 evolution is actively re-established. When this latter observation was first recorded (3), it was interpreted as renewed decarboxylation activity resulting from a combination of enzymes, the *Propionibacterium* preparation providing the decarboxylase and the *Veillonella* preparation the enzyme necessary for conversion of the "C_1" unit to CO_2.

[1]Manuscript received August 21, 1961.
Contribution from the Division of Bacteriology, College of Agriculture, Cornell University, Ithaca, N.Y.

This report describes experiments in which we have investigated by direct measurement of CO_2 evolution the phenomenon of renewed CO_2 evolution by combined extracts of *P. pentosaceum* and *V. alcalescens*.

Materials and Methods

Cultural Methods

P. pentosaceum E214 was grown in a broth medium (pH 7.0) consisting of 0.5% yeast extract, 0.5% glucose, 0.5% tryptone, and 0.5% K_2HPO_4. Following incubation at 30° C for 36 hours, the cells were harvested by centrifugation, washed once with distilled water, and stored at −20° C.

The culture of *V. alcalescens*, a recent isolate from the rumen of a sheep, was grown in a broth medium (pH 6.8–7.0) consisting of 1.0% yeast extract, 1.0% tryptone, 2.0% sodium lactate, and 0.01% cysteine. Cells were harvested after 36 hours' incubation at 37° C, washed, and stored as with *P. pentosaceum*.

Preparation and Treatment of Cell-free Extracts

Extracts of both organisms were prepared in the same manner. A heavy cell suspension (50 mg/ml) in 0.1 *M* phosphate buffer (pH 7.0) and 0.001 *M* cysteine was treated in a French pressure cell. The fragmented preparations were centrifuged at 15,000 × *g* for 30 minutes to remove cell debris and the cell-free extracts were stored in the dark at −20° C.

Dialysis of cell-free preparations was carried out against a solution of 0.02 *M* tris(hydroxymethyl)aminomethane buffer (pH 7.0) and 0.005 *M* cysteine for 24 hours at 0 to 2° C with changes of dialyzing solution after 8 and 16 hours.

Manometric Techniques

Measurement of CO_2 evolved in the decarboxylation was performed by the usual Warburg manometric methods. Each vessel contained cell-free extract, 1.0 μmole ATP, 0.2 μmole CoA, 20 μmoles succinate, and 0.1 *M* phosphate buffer (pH 5.5) in a volume of 3.0 ml. All experiments were conducted at 30° C under an atmosphere of nitrogen. External illumination was kept at a minimum.

Results and Discussion

If renewed CO_2 evolution arises as a result of the combination of enzymes supplied by *P. pentosaceum* extract and aged *V. alcalescens* extract, destruction of the protein material in either preparation should eliminate CO_2 evolution. Heat treatment of a *Veillonella* extract at 100° C for 10 minutes destroyed the ability to evolve CO_2 when the extract was combined with an untreated *Propionibacterium* extract, thus confirming the requirement for enzymes present in the extract of *V. alcalescens*. However, heat treatment of the *P. pentosaceum* extract followed by removal of the denatured protein left a clear supernatant that was as active as had been the unheated preparation (Fig. 1). These observations suggest that the stimulatory factor(s) in the extract of *P. pentosaceum* is non-enzymatic.

The non-enzymatic nature of the stimulatory factor(s) in *P. pentosaceum* was further demonstrated by dialysis of a preparation of this organism. After dialysis, stimulation of an aged *Veillonella* extract was greatly reduced (Fig. 2).

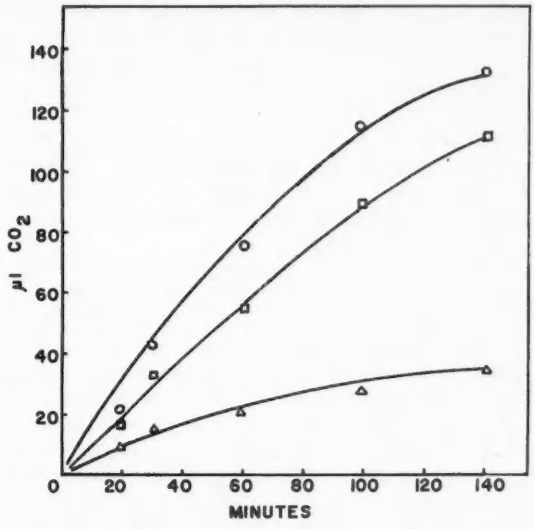

FIG. 1. Stimulation of aged *Veillonella* extract by heated and unheated *Propionibacterium* extract: ○ aged *Veillonella* + unheated *Propionibacterium;* □ aged *Veillonella* + heated *Propionibacterium;* △ aged *Veillonella.*

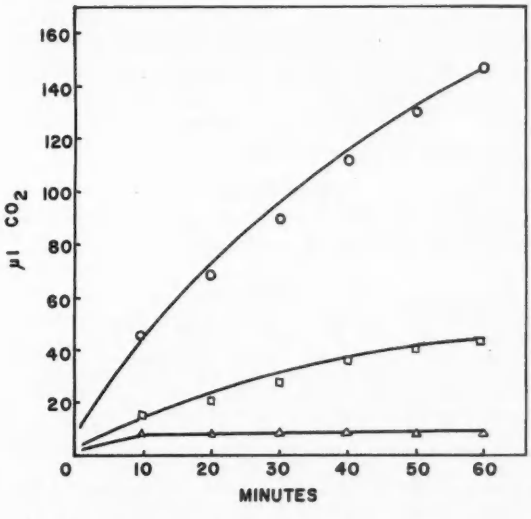

FIG. 2. Effect of dialysis upon stimulatory activity of *Propionibacterium* extract: ○ aged *Veillonella* + heated *Propionibacterium;* □ aged *Veillonella* + dialyzed heated *Propionibacterium;* △ aged *Veillonella.*

The dialyzate, when concentrated to a small volume under vacuum at 50° C, was as active as the original heated extract in stimulating decarboxylase activity of the aged *Veillonella* preparation. The heat stable stimulatory factor(s) in *P. pentosaceum* could not be replaced by the known cofactors CoA, ATP, or Mg^{++}.

When combined extracts of both organisms or an active preparation of *Veillonella* alone was treated with the biotin inhibitor avidin, CO_2 evolution was completely eliminated. This inhibition was overcome by pretreatment of the avidin with biotin. Aged extracts of *V. alcalescens*, however, cannot be reactivated by the addition of biotin.

The discovery that a vitamin B_{12} coenzyme is required for the isomerization of succinyl-CoA to methylmalonyl-CoA in *P. shermanii* (6, 7) suggested that a vitamin B_{12} coenzyme might also be present in the isomerase of *V. alcalescens*. The cobamide coenzymes are known to be light sensitive, thus a decrease of decarboxylase activity following exposure of a *Veillonella* preparation to illumination would be compatible with the participation of a B_{12} coenzyme. Exposure of such a preparation to white light illumination for 60 minutes at 0–2° C resulted in complete inactivation of the decarboxylase system. We have been unable to reactivate such illuminated systems by the addition of a *Propionibacterium* preparation or with dimethylbenzimidazolylcobamide coenzyme (DBC). Similarly DBC is without effect on aged extracts of *V. alcalescens*. Illumination of the supernatant of a boiled *P. pentosaceum* preparation does not affect its stimulatory activity. Thus, although a light sensitive B_{12} coenzyme appears to be functioning in the *Veillonella* decarboxylase system, this does not appear to be the stimulatory factor present in *P. pentosaceum*. It is possible that a specific form of cobamide coenzyme other than DBC is the stimulatory factor involved, although the failure of illumination to affect the activity of boiled *Propionibacterium* preparations renders this explanation somewhat unlikely. It is also possible that a form of biotin other than the free form is participating or that a previously unsuspected and structurally unknown factor is involved.

Acknowledgments

We wish to thank Drs. H. A. Barker and D. Perlman for gifts of 5,6-dimethylbenzimidazolylcobamide coenzyme.

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FAT PRODUCTION FROM CANE MOLASSES BY PENICILLIUM SPINULOSUM: A STUDY ON SUBSTANCES IN CANE MOLASSES INTERFERING WITH FAT PRODUCTION[1]

A. W. KHAN[2] AND T. K. WALKER

Abstract

Production of fat by *Penicillium spinulosum* in cane molasses was poorer than in a defined medium containing sucrose and inorganic salts. The fat content of dry felt obtained in defined medium was 63.8% and the fat coefficient 16.1. In molasses medium the fat content of felt dropped to 24.4% and the fat coefficient to 6.2. Removal of inorganic materials from the molasses increased fat yields substantially, and the addition of molasses ash to the defined medium reduced yields to about the same level as in untreated molasses.

Introduction

The conditions necessary for mycological formation of fat in a defined medium containing inorganic salts and glucose or sucrose in shake flasks (2) and on pilot plant scale (3) are well established. Since the economic production of fat necessitates the use of a cheap sugar source, the object of the present enquiry was to determine whether cane molasses could be used economically as a substrate for *Penicillium spinulosum*, a powerful fat-forming mold strain in shake cultures. Preliminary experiments had shown that cane molasses is inferior to glucose or sucrose as a source of carbohydrate and a study was therefore made to determine the nature of the substances in molasses depressing the fat synthesis. The results are reported in this paper.

Materials and Methods

Organism and Preparation of Inoculum

The strain of *Penicillium spinulosum* used was isolated at Manchester College of Science and Technology, Manchester, England, and its identity was confirmed at the Centraalbureau voor Schimmelcultures, Baarn, Holland (3). The culture was maintained on wort-agar by fortnightly transfer. To obtain spores for inoculation, test tubes containing 0.06–0.08 g of wheat bran moistened with 0.1 ml of 1% glucose solution were inoculated with mold spores from wort-agar tubes and incubated at 26° C for 7 days.

Media

(*a*) The defined medium of Garrido, Gad, and Walker (2) was employed. It contained (g/l.): $Na_2HPO_4.2H_2O$, 0.180; $MgSO_4.7H_2O$, 0.060; K_2SO_4, 0.055; $ZnSO_4.7H_2O$, 0.013; $FeCl_3.6H_2O$, 0.040; NH_4Cl, 1.600; and sucrose, 100 (pH 6.8 adjusted with NaOH). Tests were made with the defined medium as such and after the addition of material separated from molasses as follows.

[1]Manuscript received August 18, 1961.
Contribution from the Department of Industrial Biochemistry, Manchester College of Science and Technology, Manchester, England.
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(i) Ether and *n*-butanol extractable materials: A neutral solution of molasses was first extracted with ether (or with *n*-butanol) in a liquid–liquid extractor. The solution was then rendered acid (pH 2.0) with hydrochloric acid and re-extracted with the same solvent. The ether and *n*-butanol extracts were evaporated to dryness separately and materials obtained from 150 g molasses were added to 1 liter of defined medium.

(ii) Inorganic materials: Molasses was ashed in a muffle furnace at 500–550° C and the ash dissolved in minimum quantity of hydrochloric acid (A.R. Grade). The insoluble materials were discarded. An amount of ash solution corresponding to 150 g of molasses was added for every liter of defined medium.

(*b*) The cane molasses employed was obtained from British West Indies and was used untreated and after the following treatments.

(i) Removal of neutral volatile materials: A 30% (w/v) aqueous solution of molasses was distilled at a pH of 7.5–8.0.

(ii) Removal of volatile acids: The organic acids were removed by steam distilling a 30% (w/v) aqueous solution of molasses at a pH of 2.0 adjusted with sulphuric acid according to Clark and Hilling (1).

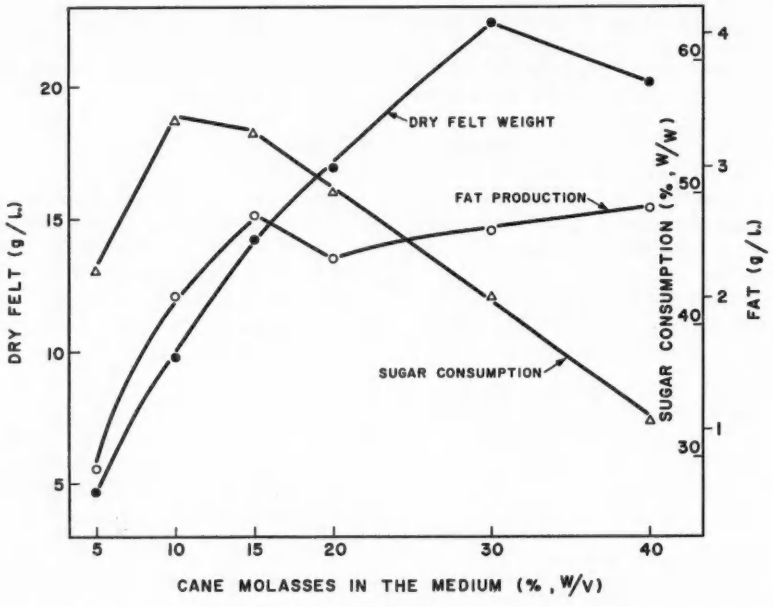

FIG. 1. Effect of concentration of cane molasses on fat formation, dry felt weight, and sugar consumption, by *P. spinulosum* (total sugar contents of molasses, 53.8 g/100 g; incubation time 6 days).

(iii) Treatment with ion exchange resin: An aqueous solution of molasses was passed through a column of cation exchange resin (Zeo-Karb 315; Permutit Company Limited) and through an anion exchange resin (De-Acidite E, Permutit Company Limited). The molasses solution was used as substrate after it was supplemented with inorganic salts necessary in defined medium.

Molasses (as such or after treatment) was diluted to 15% (w/v) with distilled water (80 g/l. sugar contents expressed as glucose) and the pH adjusted to 6.8 with sulphuric acid. This medium was found to give best fat yields with efficient sugar consumption in preliminary tests (Fig. 1). In media containing more than 15% molasses growth was stimulated with no increase in fat production and the fat coefficients were lowered substantially.

Growth Conditions and Analytical Methods

Experiments were carried out in 250-ml conical flasks, each containing 25 ml of the required medium. The surface-to-volume ratio given by this volume of substrate gave best yields of fat-rich mycelium (Fig. 2). Growth interfered with agitation in smaller volumes. The flasks were sterilized in steam at 10 lb pressure for 15 minutes, inoculated, and incubated at 26° C. For shaking, a rotary table shaker operated at 148 r.p.m. was employed. After 5, 6, 7, 8, and 9th day of incubation, eight flasks were selected at random and each of the two samples obtained by combining contents of four flasks

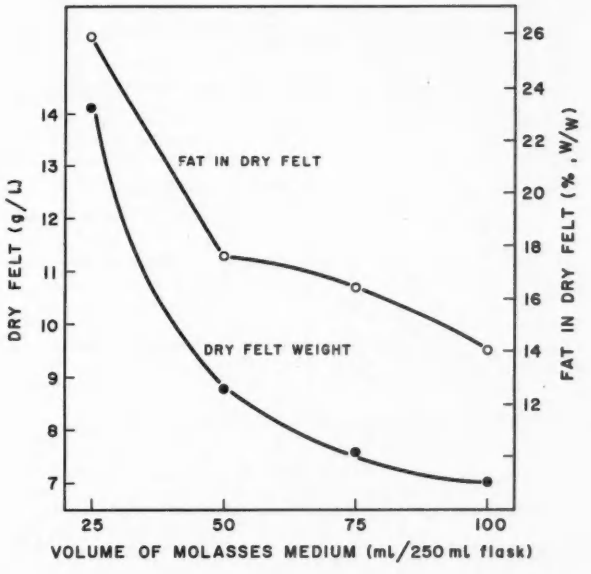

FIG. 2. Effect of volume of cane molasses medium per flask on fat formation by *P. spinulosum* (initial sugar content, 80.3 g/l.; incubation time 6 days).

were analyzed for pH, total sugars (as glucose), weight of the dry felt, and fat in the dry felt as described by Woodbine, Gregory, and Walker (5).

Fat coefficients were calculated from the weight of fat produced per 100 g of sugar consumed.

Results and Discussion

Fat production in untreated molasses and in defined medium are compared in Table I. The removal of materials of organic nature, viz., neutral volatile ingredients and volatile acids present in molasses as a result of bacterial oxidation and breakdown product of sugars (4), did not improve fat yields (Table II), but the removal of minerals by ion exchange resin did give higher fat yields (Fig. 3).

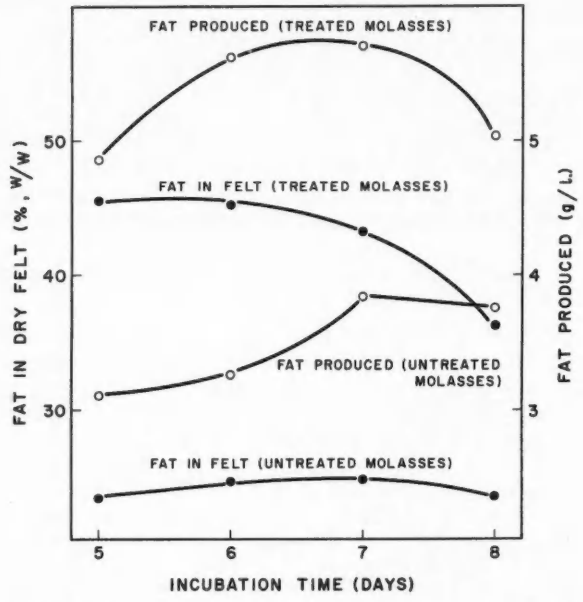

FIG. 3. Effect of treatment of cane molasses with ion exchange resins on fat formation by *P. spinulosum*.

TABLE I

Comparison of fat formation in defined medium and cane molasses medium by *P. spinulosum*

	Defined medium				Cane molasses medium			
Days of growth	6	7	8	9	6	7	8	9
Dry felt (g/l.)	8.8	11.0	13.8	14.9	14.4	15.7	15.7	17.1
Fat in dry felt (%, w/w)	63.8	62.1	60.7	57.2	24.3	24.4	23.6	23.8
Fat (g/l.)	5.6	6.8	8.4	8.5	3.2	3.8	3.9	4.0
Sugar consumption (g/l.)	48.4	50.8	52.1	53.2	52.6	61.2	70.1	79.1
Fat coefficient	11.7	13.4	16.1	16.0	6.0	6.2	5.5	5.0

The addition of ether and *n*-butanol extracted materials to the defined medium decreased fat yields to some extent and the addition of molasses ash reduced fat formation in the defined medium to about the same level as in untreated molasses (Table III). These results indicate that fat production from cane molasses may be feasible if an economical process can be devised for the removal of undesirable minerals.

TABLE II

The effect of removal of volatile materials from molasses on fat formation by *P. spinulosum* (Incubation time 6 days)

	Dry felt (g/l.)	Fat		Sugar consumption (g/l.)
		in dry felt (%, w/w)	g/l. of medium	
Cane molasses as such	12.6	22.9	2.9	27.8
Cane molasses, neutral volatile substances removed	10.6	24.3	2.6	29.9
Cane molasses, volatile acids removed	8.4	22.5	1.9	23.6

TABLE III

Effect of addition of various molasses fractions to the defined medium on fat formattion by *P. spinulosum* (Incubation time 6 days)

	Dry felt (g/l.)	Fat		Sugar consumption (g/l.)
		in dry felt (%, w/w)	g/l. of medium	
Defined medium	8.8	63.8	5.6	48.4
Defined medium + ether extract	9.3	52.5	4.9	37.7
Defined medium + butanol extract	8.8	49.1	4.3	32.0
Defined medium + ash from molasses	9.0	23.3	2.1	27.1
Cane molasses medium	12.6	22.9	2.9	27.8

Acknowledgment

The authors wish to express their thanks to Dr. B. H. Arnold, Fermentation Research Division, Glaxo Laboratories Ltd., Stoke Poges, England, for his help in the work.

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LYSOGENY IN ENTEROPATHOGENIC ESCHERICHIA COLI

I. GENERAL CHARACTERISTICS OF BACTERIOPHAGES ISOLATED FROM O26:B6 AND O111:B4 SEROTYPES[1]

W. Robert Bailey and John P. Glynn[2]

Abstract

Twenty strains each of *Escherichia coli* O26:B6 and O111:B4 were tested for lysogeny. Of these, 30% of the former were lysogenic but none of the latter showed this characteristic when tested by the same criteria. Colicin-like activity was initially mistaken for phage activity when O26:B6 phage preparations were tested on O111:B4 strains. The activity of the O26:B6 phages appeared to be restricted to this particular somatic group. High titers of phage could not be obtained by orthodox methods.

Introduction

Routine typing and epidemiological studies of enteropathogenic *Escherichia coli* would be facilitated greatly if pure factor antisera were available for the various B-antigen complexes. At the present time, all attempts to produce such antisera by normal immunization and agglutinin–absorption procedures are inapplicable, due to the heat stability of the agglutinin-binding capacity of the B-antigen complex, the group specificity of the B-antigen complexes, and the failure to isolate B-minus strains either in the field or by laboratory procedure.

It occurred to the authors that if bacteriophage could be used effectively as a mediator of genetic exchange in transduction procedures for the development of bacterial hybrids in the genus *Salmonella*, it might also be applicable in attempts to produce hybrids in *E. coli*. If hybrids could be thus developed, they could then be used in producing the appropriate antisera.

Pursuing this approach, two serotypes, O111:B4 and O26:B6 were selected and a search initiated for a phage with a host range which encompassed both serotypes. Nicolle *et al.* (3, 4) isolated several phages which, reportedly, exhibited group specificity for O111:B4 and O26:B6 respectively. The authors were unable to propagate these phages on the 'sensitive' strains, and it was decided to attempt the isolation of additional phages from lysogenic strains of O26:B6 and O111:B4.

Experimental Procedures

Source of Strains

Twenty strains each of O111:B4 and O26:B6 serotypes, isolated from cases of gastroenteritis, were obtained from Dr. W. H. Ewing of the Communicable Disease Center, Atlanta, Georgia. To facilitate expression, *E. coli* types O111:B4 and O26:B6 hereafter will be abbreviated O111 and O26 respectively.

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The strain of each type will be indicated by a numeral in parenthesis immediately following the somatic group designation, i.e., O26:B6 strain 2 will be written O26(2).

Incidence of Lysogeny

A modification of Fisk's method (2) was used to test the total of 40 strains for lysogeny. The isolation procedure consisted of culturing each strain, O26 or O111, with each of the remaining 19 strains for 12 to 14 hours in Trypticase Soy (TS) Broth (Baltimore Biological Laboratories). The cultures were centrifuged and the activity of each supernatant was tested against the two strains used. The activity was determined by placing a drop of young culture (4–5 hours) on the surface of a peptone agar plate, allowing time for the drop to dry thoroughly, and then placing a drop of supernatant on the same area. After the plates were dry, they were incubated at 37° C for 3–5 hours, and examined. A further examination was made after overnight refrigeration.

Since lysogenic strains are normally resistant to the phage they carry (1), lytic activity of the supernatant on one of the pair of strains was taken as an index of lysogeny in the other. The results of screening the twenty O26 strains for phage activity indicated that 30% or six of the strains were lysogenic. The lysogenic strains and their propagating strains are shown in Table I.

The same procedure was used to screen the O111 strains for lysogeny, but none of the latter strains proved to be lysogenic by these criteria.

TABLE I

Lysogenic and sensitive strains of *Escherichia coli* O26:B6 from human sources

Lysogenic strain	Propagating strain
3	2
4	2
5	2, 12, 13
8	2, 5
12	15, 18
13	15

General Characteristics of the Phages

Host Range

The phages from the lysogenic strains were propagated on sensitive strains and the pattern of their activity on the O111 and O26 strains was determined. The critical test concentrations (CTC), i.e. the highest dilution which gave confluent lysis with the sensitive strain, was used for each phage. The activity was determined by the spotting procedure described previously. The data, summarized in Table II, suggest that there are several distinctly different phages, and that their activity is rather broad within the O26 strains tested. Initial observations suggested that some of the O26 phage preparation were active on several O111 strains. Further study, however, revealed that the activity on O111 strains was due not to phage, but to a colicin-like substance elaborated by O26(2). This was clearly indicated when the cell-free supernatant of a 24–48 hour culture of O26(2) was spotted on the test strains. The spotted areas exhibited marked growth inhibition. Since the lysates were prepared

TABLE II

Host range of O26 lysates on O26:B6 strains

Lysates	Strains tested															
	1	2	5	6	7	8	9	10	11	12	13	15	16	17	18	20
3/2	TNTC	CL	CLSG	TNTC	TNTC	—	CLSG	TNTC	—	—	—	—	CLSG	TNTC	PL	TNTC
4/2	—	CLSG	TNTC	—	—	—	—	—	—	—	—	—	—	—	—	—
5/2	—	CL	—	—	—	—	—	—	—	PL	PL	PL	—	—	—	—
8/2	—	TNTC	TNTC	—	—	—	—	—	—	—	—	—	—	—	—	—
8/5	TNTC	CL	CLSG	TNTC	TNTC	—	CLSG	TNTC	—	—	—	—	CLSG	TNTC	PL	TNTC
5/12	—	CL	—	—	—	—	—	—	—	CL	CL	CL	—	—	—	—
5/13	—	CL	—	—	—	—	—	—	—	CL	CL	CL	—	—	—	—
12/15	—	CLSG	TNTC	—	—	—	CLSG	—	CLSG	—	—	CLSG	—	—	TNTC	—
13/15	—	CLSG	CLSG*	CLSG*	—	CLSG*	CLSG	CLSG*	CLSG*	—	—	CLSG*	CLSG*	CLSG*	CLSG*	—
12/18	—	TNTC	TNTC	—	—	—	TNTC	—	TNTC	—	—	—	—	—	TNTC	—

NOTE: Strains 3, 4, 14, and 19 were not sensitive to any phage and are not included in the table.
LEGEND: The first number in the numerical designations for the lysates indicates the strain from which the phage was isolated and the number following the oblique line indicates the propagating strain.

TNTC Too numerous to count.
CL Confluent lysis.
CLSG Confluent lysis with secondary growth.
PL Plaques.

*Denotes heavy secondary growth.

by propagation in broth medium and O26(2) was colicinogenic, both principles, colicin and phage, were present in the same preparation. Table II, therefore, shows the activity of the lysates on O26 strains only, but it must be recognized that the activity recorded for lysate 13/15 may not be due entirely to phage. Furthermore, differences in host range, exhibited by certain phages following their propagation on different strains, may be the result of partial adaptation.

Since both principles could be present, it was necessary to distinguish between lysogeny and colicinogenicity. It was observed that a differentiation could be made on the following bases: (*a*) only the phage could be propagated on the sensitive strain; (*b*) phage activity decreased uniformly with lysate dilution, but colicin activity ceased abruptly at a dilution of approximately 10^{-2}; (*c*) when both were present in a broth preparation, the phage could be removed by absorption with cells of a sensitive strain, while colicin activity was unaffected by this procedure.

Propagation

One problem with these *E. coli* phages was low titer. It was extremely difficult to obtain lysates with a titer higher than 10^6 plaques per ml. As all of the phages were active on O26(2), the latter appeared to be the logical propagating strain, but extensive efforts, utilizing several propagation methods, failed to yield high-titered lysates. Furthermore, other sensitive strains tested proved no better.

Plaque Types

Most of the phages produced small, irregular, turbid plaques (plaques showing secondary growth). The turbidity, which appeared rapidly, was especially evident in the lower dilutions, which contained sufficient phage to give confluent lysis.

Peptone agar, on which the sensitive strain grew less profusely, was used in place of TS agar to retard bacterial development and thus prevent the plaques from being overgrown prior to counting. The only exception was the phage carried by O26(5). This phage produced larger, clearer plaques and normally exhibited a higher titer than the others.

Discussion

The only reports concerning phages isolated from enteropathogenic *E. coli* strains are those of Nicolle *et al.* (3, 4), who isolated several phages which would lyse O111:B4, O26:B6, and O55:B5 serotypes. Their data seemed to indicate that the activity of any one phage appeared to be confined to a particular somatic group. To date we have been unable to find any reports of *E. coli* phages attacking more than one somatic group. It should be recognized that phages which are group specific would be far more useful as epidemiological tools than phages with a wide host range. The latter type of phage would be more suitable for transduction procedures.

Although in our study only six phages were isolated from the twenty O26 strains, their host range was limited to the somatic group of the strains from which they were isolated. All proved to be inactive on the O111 strains. This would suggest, therefore, that the phage receptor sites on the bacterial cell are probably closely associated with the specific group antigens.

In the initial phases of this investigation, the colicin activity exhibited by some strains led to some early misinterpretation and confusion. Other investigators should be cautioned about confusing colicin activity with phage activity. Although the data are not presented, several of these O26 and O111 strains produced colicins. In general, the colicins had a broader range of activity than most of the phages, but lost their ability to suppress growth visibly when diluted beyond 10^{-2}. In screening for lysogeny, therefore, it was necessary to dilute the lysates at least this much. Our inability to obtain fairly high-titered lysates with these phages might be due to small burst sizes and long latent periods. A phage, isolated from an O111 strain (paper II of this series), was observed to have an average burst size of about 36 and a latent period of approximately 40 minutes. Phages with such characteristics are difficult to obtain in high titer by normal propagation procedures. The propagation difficulties experienced with the phages reported here would indicate that they also have similar burst sizes.

It was noted earlier that O26(2) was sensitive to all the phages isolated during the investigation. The authors did not isolate any phage from any of the enteropathogenic serotypes which would not lyse this O26 strain. The latter, therefore, appears to be an excellent strain for screening O26 strains for lysogeny.

Due to the fact that the phages isolated in this study were group specific, they obviously would be unsuitable for mediating transduction between the O111 and O26 strains used, since the basic requirements for this phenomenon were not fulfilled.

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LYSOGENY IN ENTEROPATHOGENIC ESCHERICHIA COLI

II. CHARACTERIZATION OF E. COLI BACTERIOPHAGE D-1[1]

JOHN P. GLYNN[2] AND W. ROBERT BAILEY

Abstract

One of 20 strains of *Escherichia coli* O111:B4 was found to be lysogenic for a phage which showed activity on several O26:B6 strains. High titers of this phage were obtainable only by UV induction. Normal propagation procedures failed to provide a desirable yield of phage. The calculated burst size of this phage was approximately 36 particles per cell. Other characteristics of the phage, designated D-1 by the authors, are given. Its potentiality as a possible transducing agent was also investigated. Some evidence is offered for the suspected site of attachment on the host cell.

Introduction

While investigating phage and colicin production in several *E. coli* strains of serotypes O111:B4 and O26:B6, a lysogenic strain of O111:B4, which harbored a bacteriophage that was active on a strain of O26:B6, was discovered. The lysogeny of this particular strain was not detected initially (3) due to the fact that the harbored phage was inactive on O111:B4 strains. This phage, designated D-1 (Delaware) by the authors, however, was active on several O26:B6 strains.

The original purpose of investigating lysogeny in the enteropathogenic *E. coli* was to find a phage which could be used as a potential transducing agent between strains of serotypes O26:B6 and O111:B4. On the basis of its host range, the D-1 phage appeared to offer some hope of accomplishing this purpose.

In an extensive series of transduction experiments, where selection was made for such transferred characters as motility, streptomycin resistance, and B-type antigens, no positive results were obtained. This failure to obtain transductants, together with the propagation difficulties experienced with other phages from enteropathogenic *E. coli* strains (3), prompted the authors to attempt a more complete characterization of the D-1 phage.

Materials and Methods

The enteropathogenic strains of *E. coli* and their designations are identical with those described previously (3).

All cultures were grown in Trypticase Soy Broth (TS) (Baltimore Biological Lab.) at 37° C. Counts of viable bacteria were determined by the "drop plate" method (8) using calibrated pipettes.

L'woff *et al.* (5) induced lysogenic cells to produce phage by ultraviolet (UV) irradiation. The authors used this method to obtain D-1 phage from its lyso-

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genic host. Preliminary to the induction procedure, a pure line of O111(15) cells was obtained from a streptomycin-resistant variant by single cell isolation, utilizing a Bausch and Lomb micromanipulator.

The UV induction procedure was patterned after that of Lederberg and Lederberg (4). A 10-ml aliquot of a suspension of O111(15), containing 9×10^8 cells per ml, was centrifuged and the pellet resuspended in 5 ml of 0.5% saline. This suspension was transferred to a Petri dish on a rotary type shaker, and irradiated with a 15-watt GE germicidal lamp for 50 seconds at a distance of 50 cm. Immediately following irradiation, the cells were diluted in an equal volume of double strength TS broth and incubated at 37° C. The entire procedure was carried out in the dark. A maximum phage yield of 10^8–10^9 per ml was obtained after 3 hours' incubation, while unirradiated aliquots yielded only 10^2–10^3.

The phage titer was estimated by plaque count. One milliliter of a 4–5 hour culture of O26(2) was used for seeding each plate in preparing the 'lawn'. The excess of culture was removed with Pasteur pipettes. When the seeded surface of the plates was dry, four drops of each dilution 10^{-4}, 10^{-5}, and 10^{-6} were applied with dropping pipettes calibrated to deliver 0.01 ml per drop. After incubation at 37° C for 3–4 hours, counts were made directly or after overnight refrigeration. Lysates were freed of bacteria by filtration, as repeated tests clearly indicated that D-1 phage was thermolabile, being 90% inactivated in 10 minutes at 60° C. Ultrafine membrane filters retained a high percentage of the phage, while an L-3 Seitz filter proved highly satisfactory. Sterility of the lysate was checked by inoculating 1 ml into 10 ml TS broth and incubating at 37° C for 48 hours.

The resulting plaques were small, irregular, and turbid, resembling those previously reported for other phages of enteropathogenic *E. coli* origin (3).

Experimental Procedures

Host Range

Preliminary results had indicated that supernatants from unirradiated cultures of O111(15) were active on both O26 and O111 strains. More extensive screening was carried out with a critical test concentration (CTC) (3) of a D-1 phage lysate, determined from its activity on O26(2). The results obtained with 20 strains each of O26 and O111 are shown in Table I, with a brief description of the type of activity observed. Because of the difficulty encountered with colicins (3), every strain on which some activity was observed was tested for its ability to absorb phage D-1.

Sufficient phage was added to 2-ml aliquots of the absorbing cell suspensions to give a resultant concentration of 10^4 particles per ml, and the mixture incubated at 37° C for 15 minutes. Absorption was followed by centrifugation and assay for free phage. The results in Table II indicated that the O111 strains did not absorb appreciable quantities of phage, from which it may be inferred that the original activity observed on these strains was not due to phage. It may be concluded, therefore, that O111(15) is colicinogenic as well as lysogenic.

TABLE I

Activity of D-1 lysate on several O26:B6 and O111:B4 strains of *Escherichia coli*

Strain	Type of activity observed with the CTC concentration of D-1
O26(1)	Definite plaque formation, but much less than standard lysis
O26(2)	Standard lysis
O26(3)	Standard lysis
O26(4)	Standard lysis
O26(16)	Definite plaque formation, but much less than standard lysis
O26(17)	Definite plaque formation, but much less than standard lysis
O111(1)	Slight inhibition
O111(2)	Slight inhibition
O111(3)	Slight inhibition
O111(4)	Strong inhibition, spreading beyond the drop area
O111(5)	Strong inhibition
O111(6)	Strong inhibition
O111(7)	Slight inhibition
O111(8)	Slight inhibition
O111(9)	Strong inhibition
O111(13)	Slight inhibition
O111(14)	Strong inhibition
O111(17)	Strong inhibition
O111(18)	Strong inhibition

NOTE: Strains on which no activity was observed are omitted from the table.
LEGEND: Standard lysis—degree of lysis observed when CTC concentration is spotted on O26(2).
Strong inhibition—slight growth in drop area.
Slight inhibition—definite growth in drop area.

TABLE II

Phage D-1 absorption by strains of *E. coli* O26:B6 and O111:B4. The phage control contained 114×10^2 plaque formers per ml

Strain	Free phage/ml	% absorption
O26(1)	$<10^2$	>99
O26(2)	$<10^2$	>99
O26(3)	$<10^2$	>99
O26(4)	$<10^2$	>99
O26(16)	$<10^2$	>99
O26(17)	$<10^2$	>99
O111(1)	112×10^2	2
O111(2)	108×10^2	5
O111(3)	111×10^2	3
O111(4)	108×10^2	5
O111(5)	111×10^2	3
O111(6)	112×10^2	2
O111(7)	111×10^2	3
O111(8)	114×10^2	0
O111(9)	108×10^2	5
O111(13)	109×10^2	4
O111(14)	108×10^2	5
O111(17)	117×10^2	0
O111(18)	104×10^2	9

Site of Attachment of D-1 to E. coli O26(2) Cells

During the course of the investigation, it was observed that the supernatants of 24–48 hour cultures of O26(2) contained demonstrable quantities of B antigen. This could be demonstrated by the precipitin reaction, using the supernatant of O26(2) as precipitinogen and O26(OB) antiserum as the

precipitin. Since phage D-1 would lyse strains possessing a B-type antigen, it was decided to determine if this phage was absorbed or neutralized by the B-antigen complex.

The following procedure was used: cells from 24 hour cultures on TS agar were harvested in 0.5% saline. The saline suspension contained approximately 3×10^{10} cells per ml. A 10-ml portion of the suspension was mixed with glass beads and shaken vigorously for 30 minutes. The suspension was then centrifuged and the supernatant Seitz-filtered.

The filtered extract was treated for 2 hours at 37° C, with O26 O antiserum (0.1 ml per ml) to combine with any O antigen which might be present. A scant precipitate developed after overnight refrigeration. This precipitate was spun down and the decanted supernatant treated with O26(OB) antiserum (0.1 ml per ml) for 2 hours at 37° C. A heavy flocculent precipitate was observed in the second absorption which indicated that substantial quantities of B antigen were present. Titration of the supernatant revealed a considerable reduction in the titer of the B antibody. This result tended to confirm further that B antigen was present in the original extract.

The untreated extract, the O absorbed portion, and the OB absorbed portion were tested for their phage-neutralizing capacity. In each instance, the neutralizing capacity was determined by mixing the phage with a portion of the extract and incubating the mixture at 37° C for 1 hour. An equal volume of TS broth served as the control.

The results, as shown in Table III, indicated that the initial extract neutralized most of the phage. After absorption with O antiserum, however, the extract lost the ability to neutralize the phage, even though an abundance of B antigen was still in solution. This result strongly suggested that the site of attachment of phage D-1 was not in the B antigen complex, but probably somewhere in the O antigen complex.

TABLE III

The neutralizing effect of soluble O antigen on phage D-1

Control, free phage/ml	Antigen extract	Free phage after treatment 1 hr at 37° C	Neutralization, %
97×10^5	Unabsorbed	6×10^3	>99
148×10^5	Absorbed with O26(O) antiserum	134×10^5	9
188×10^5	Absorbed with O26 (OB) antiserum	163×10^5	13

Single Step Growth Experiment

Using the technique developed by Ellis and Delbruck (1), estimates of the burst size and latent period of D-1 were obtained. The initial cell count was 6.8×10^8 per ml, while the initial phage count was 18×10^8 per ml. The phage lysate was diluted 1:10 with the cell suspension and absorbed at 37° C for 10 minutes. The free phage remaining after this period was calculated to be 0.4×10^7 per ml, indicating 97.8% absorption. The multiplicity of infection, which implies the average number of phage particles per cell, was calculated from the data and found to be 0.29.

TABLE IV

Plaque counts on the assays from the first and second growth tubes of the burst size determination

Time (min)	FGT, plaques/0.01 ml	SGT, plaques/0.01 ml
20	123	
25	121	
30	119	
35	123	
39	134	
41	146	
43	227	
45	TNTC	3.5
50		10.8
55		21.0
60		27.3
65		28.5
70		30.4
75		36.5
80		39.8
85		39.0
90		43.0
100		42.3
110		42.0

LEGEND: FGT First growth tube. SGT Second growth tube. TNTC Too numerous to count.

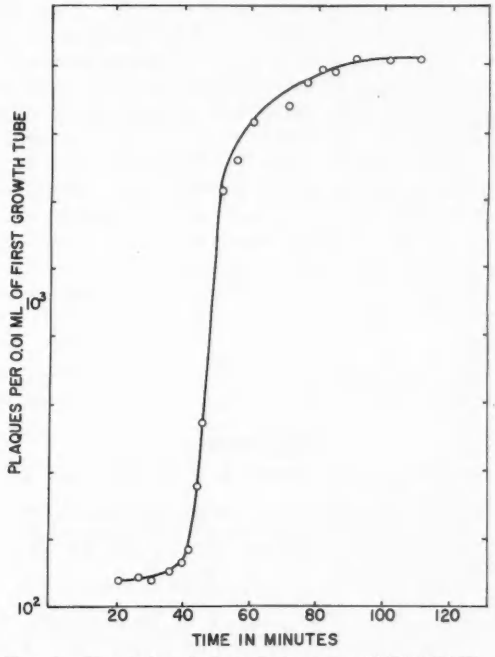

FIG. 1. Burst size of phage D-1 on *E. coli* O26:B6(2).

The results in Table IV show the average plaque counts at various intervals during the propagation cycle. The average plaque count, after lysis was complete, in the second growth tube was 42.4. This value when multiplied by 10^2 represents a count of 4240 in the first growth tube. The average plaque count during the latent period, after correcting for free phage, was 117. The average burst size would be, therefore, 4240 divided by 117, which equals 36, and the latent period appeared to be 39–40 minutes.

Figure 1 shows the rise period to be approximately 50 minutes.

Discussion and Conclusions

Previous work (3, 6, 7) suggested that the host range of phages which lysed enteropathogenic *E. coli* strains was confined to a particular somatic group. The results of Nicolle *et al.* might reflect the type of isolation procedures used, since their interest was focussed primarily on phages as epidemiological tools. Although the D-1 phage lysed only strains belonging to the O26 somatic group, its host range must encompass some O111 strains since it was isolated from a lysogenic O111 strain.

The site of attachment of this phage was demonstrated to be other than the B6-antigen complex in O26. This was taken as an indication that the phage was not a B type. At the time of writing, there are no accounts of similar studies with phage which lyse strains possessing a B-type antigen. Toft (9), however, worked with *E. coli* strains possessing L and A varieties of K antigens. He isolated phage, of which some attacked only capsular forms, some only non-capsular forms, while others lysed both. It is conceivable, therefore, that D-1 phage might attack B-minus forms of O26 (presently unobtainable) and strains of other somatic groups which lack this type of envelope antigen.

The authors had encountered difficulty in attempting to propagate several enteropathogenic phages, in spite of good plaque development on the strain used for propagation (3). The D-1–O26(2) system behaved in the same manner. While admittedly the properties of the above system could be altered by a change in the environment, the characteristics of the system are not conducive to the preparation of high-titered phage lysates. The apparent similarity between this system and the systems studied previously suggests that they might possess similar properties. The only acceptable method for the preparation of reasonably good lysates appears to be induction of the lysogenic strains by ultraviolet light.

The comparatively small plaques exhibited by phage D-1 suggest a rather large particle size, since diffusion is usually determined by particle size (2).

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LYSOGENY IN ENTEROPATHOGENIC ESCHERICHIA COLI[1]

III. THE EFFECTS OF TEMPERATURE AND CHLORAMPHENICOL ON THE LYSOGENY OF D-1 PHAGE-INFECTED CELLS[1]

JOHN P. GLYNN[2] AND W. ROBERT BAILEY

Abstract

Evidence is presented to show that cells of *Escherichia coli* O26:B6 infected with *E. coli* O111 phage D-1 exhibited a greater lysogenic response if the temperature, shortly after infection, was lowered from 37° C. Under the same conditions, lysogeny among survivors was reduced markedly at 45° C. Cooling the system to 20° C prior to infection increased both the survival rate and lysogenic response among phage-infected survivors. Appropriate treatment with chloramphenicol increased both survival rate and lysogenic frequency.

Introduction

On the basis of presumptive evidence, the authors suspected that the D-1 system (6) had a rather low frequency of lysogenization at 37° C. It has been recognized that conditions such as incubation temperature (2, 7) and the presence of chloramphenicol (3, 4) will influence the lysogenic response for some bacteriophages. Since the authors were interested in obtaining a high frequency of lysogenization in the D-1 system these conditions were investigated.

Materials and Methods

The D-1 phage was obtained from a lysogenic strain of *Escherichia coli*, O111:B4 str. 15, and the host strain used for this study was *E. coli* serotype O26:B6 str. 2. This phage–host system, and the methods for lysate preparation, plaque count, and viable cell count have been described previously (6).

The method used for determining the frequency of lysogenization was patterned after that of Bertani and Nice (2). Basically, the method involves first, the treatment of a cell population with sufficient phage to ensure that each cell becomes infected and, secondly, the plating of the population before the cells reach the end of the latent period. When the surviving cells developed into colonies, the latter were tested for lysogeny. Resistance to the lysogenizing phage was used as an index of lysogeny. This was done by picking colonies into a small amount of Trypticase Soy Broth TS (Baltimore Biological Laboratories) and incubating at 37° C for a few hours. After incubation, the cultures were assayed for phage resistance by spot testing (1) with CTC concentrations of D-1 phage.

Chloramphenicol was prepared in aqueous stock solutions (1 mg per ml) and stored under refrigeration when not in use.

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Experimental Procedures

While attempting to determine the optimal conditions for plaque formation of D-1 on O26(2), it was noticed that temperature had a decided effect on plaque development. In an experiment, designed to investigate this effect, peptone agar plates were equilibrated for 30 minutes at five different temperatures: 20° C, 25° C, 30° C, 37° C, and 45° C. A culture of O26(2), grown at 37° C was used for seeding the plates, which were allowed to dry at the respective temperatures before they were spotted with appropriate dilutions of D-1 phage. The 30° C and the 37° C plates were removed from the incubator after 3 hours. The remaining plates were incubated at their respective temperatures, and frequently observed during development of the lawns. The results, as shown in Table I, indicate that at both 20° C and 45° C, there was no phage activity. Due to their small size and the fact that they were overgrown, plaques were difficult to count at 25° C. There were no visible differences between the plaques on the 30° C and 37° C plates.

TABLE I

The effect of temperature on plaque formation

Temp. of incubation	Plaque formers per ml	EOP*
20° C	0	0
25° C	9×10^5	0.3
30° C	28×10^5	1
37° C	28×10^5	1
45° C	0	0

*EOP Efficiency of plating. Calculations based on number of plaques obtained at 37° C.

In order to ascertain whether the lack of plaques in the previous experiment was indicative of an increase in lysogeny, the technique of Bertani and Nice (2) was applied to the D-1–O26(2) system. Since plaques were not observed at either 45° C or 20° C, the frequency of lysogenization at both of these temperatures and at 37° C was determined.

A broth culture of O26(2) was grown at 37° C until a turbidity of approximately 3×10^8 cells per ml was attained. The cells were then washed and resuspended in TS broth. Concurrently, a D-1 lysate was diluted to approximately 3×10^8 particles per ml. After equilibration at 37° C, the cell suspension was diluted 1:10 with the phage lysate. A 1:10 dilution of cell suspension in TS broth served as control. After an absorption period of 5 minutes, the mixture was diluted and spotted on TS agar plates. The plates had been equilibrated for 1 hour at the appropriate temperatures. After 10 to 12 hours incubation, the plates were placed at room temperature until the colonies developed. The colonies which developed at the various temperatures were tested for lysogeny. At each temperature, 100 phage-infected survivors and 100 colonies from the corresponding control plate were tested for their phage resistance. The total of 300 control colonies were sensitive to the D-1 phage.

The results, summarized in Table II, show approximately the same percentage survival at the three incubation temperatures. Lysogeny among survivors, however, was influenced markedly by temperature, varying from 11% at 45° C to 82% at 20° C.

TABLE II

The effect of temperature on the lysogenization of *E. coli* O26(2) infected with phage D-1

Phage input*	3.3×10^8/ml
Bacterial input*	2.6×10^7/ml
Unabsorbed phage	1.8×10^8/ml
Multiplicity of infection	5.8
Infected bacteria (expected)	2.6×10^7/ml

Temp., °C	Survivors		Initial cells†	
	Following infection	% lysogenized	Survivors, %	Lysogenized, %
20	22×10^6/ml	82	8.5	6.9
37	23×10^6/ml	40	8.8	3.5
45	19×10^6/ml	11	7.3	0.8

*Figures given here have been arrived at by calculation and take into consideration the dilution factor.
†The figures in these columns are calculated from the values shown in the columns under survivors.

The degree of lysogeny was calculated on the basis of the expected infection rate, using the following formula:

$$\frac{\%\text{ lysogeny among survivors} \times \text{number of survivors}}{\text{total number of infected bacteria}} = \%\text{ lysogeny.}$$

For example, at 20° C, the percentage of lysogeny was calculated as follows:

$$\frac{82.0\ (22 \times 10^5)}{2.6 \times 10^7} = 6.9\%\text{ lysogeny.}$$

In another experiment, the procedure used above was applied to a system that had been precooled to 20° C. In this instance the absorption time was increased to 30 minutes due to a slower rate of absorption at this temperature. The results (Table III) show a significant increase in both survival and lysogeny. Cultures made from all control colonies were sensitive to the CTC of D-1.

The effect of chloramphenicol on the frequency of lysogeny in the D-1–O26(2) system was determined using the general procedure described above. A chloramphenicol concentration of 5 μg per ml was selected because preliminary tests had indicated that this concentration inhibited O26(2) cell division without decreasing the viable cell count. In one set of tubes, the chloramphenicol was added to both the phage and the cell suspension 10 minutes prior to mixing. In another set, the chloramphenicol was added 10 minutes after mixing. The phage and the cells were mixed in equal volume and zero time taken as the time of mixing. The controls consisted of an equal volume of TS broth containing 5 μg chloramphenicol per ml in lieu of phage lysate. The cell–phage mixtures were incubated at 37° C for 1 hour, then diluted and spotted on TS agar plates. The colonies which developed from the cells surviving infection were tested for lysogeny. Cultures made from the 200 colonies picked from the control plates were sensitive to phage D-1. The data, shown in Table IV, indicate that chloramphenicol treatment approximately doubled both survival and lysogeny among survivors.

TABLE III

The effect of 20° C temperature on the lysogenization of *E. coli* O26(2) infected with phage D-1

Phage input*	2.0×10^8/ml
Bacterial input*	6.2×10^6/ml
Unabsorbed phage	1.5×10^8/ml
Multiplicity of infection	8.1
Infected bacteria (expected)	6.2×10^6/ml

Unabsorbed phage/ml	Multiplicity† of infection	Estimated no. of infected cells/ml	Survivors		Initial cells	
			Following infection	% lysogenized	Survivors, %	Lysogenized, %
1.5×10^8	7.7	6.2×10^6	1.5×10^6/ml	95	24.2	23.0

*Figures given here have been arrived at by calculation and take into consideration the dilution factor.
†Determined from the number of D-1 phage absorbed by the O26(2) cells.

TABLE IV

The effect of chloramphenicol on the lysogenization of *E. coli* O26(2) by phage D-1 at 37° C

Phage input*	3.0×10^8/ml
Bacterial input*	2.5×10^7/ml

Addition of chloramphenicol	Unabsorbed phage/ml	Multiplicity† of infection	Expected no. of infected bacteria/ml‡	Survivors		Initial cells	
				Following infection	% lysogenized	Survivors, %	Lysogenized, %
10 min before mixing	19.5×10^5	12	2.5×10^7	36×10^5 per ml	77	14.4	11.1
10 min after mixing	35.5×10^5	12	2.5×10^7	44×10^5 per ml	74	17.6	13.0

*Figures given here have been arrived at by calculation and take into account the dilution factor.
†Determined from the number of D-1 phage which adsorbed to the O26(2) cells.
‡Based on zero time plate count.

NOTE: Although there is a discrepancy of 16×10^5 phage/ml in the counts of the unabsorbed phage, these figures represent less than a 1% difference in the initial phage concentration.

Discussion and Conclusions

Temperature influences the lysogenic response of O26(2) cells infected with phage D-1. If cells, infected at 37° C, are placed at 20° C during the early phase of the latent period, the frequency of lysogenization among survivors is increased. In contrast, the lysogenic response among survivors is decreased by incubating the infected cells at 45° C during this period. Under these conditions, temperature does not seem to influence the event leading to either lysis or lysogeny, since approximately the same proportion of infected cells undergo lysis at 20°, 37°, and 45° C.

When O26(2) cells are infected with D-1 at 20° C, and maintained at this temperature, both the proportion of cells surviving infection and the frequency of lysogenization among survivors is increased markedly. The increase in lysogeny, based on the expected number of infected cells at 20° C as compared with 37° C, is approximately six- to seven-fold. Bertani and Nice (2) have reported a fourfold increase in lysogeny with phage P-1 and *Shigella dysenteriae*, when the temperature was lowered from 37° C to 20° C.

Lieb (7) observed, with *lambda* phage, that low temperature favored an increase in lysogenic response among cells surviving infection, without seeming to affect the lysis versus lysogeny event in the infected cell population. This is similar to the results reported here with D-1. In addition, Lieb demonstrated that cells, lysogenic for *lambda*, could not be cured of lysogeny by growth at high temperatures. This led her to suggest that the heat-sensitive phase might be correlated with the time required for lysogeny to become established in potentially lysogenic cells. This hypothesis might apply also to our D-1 system where, although lysogeny is decreased markedly at 45° C, some of the surviving cells are lysogenic. This degree of lysogeny might represent those cells in which lysogeny became established rapidly.

The addition of chloramphenicol to suspensions of O26(2) cells, either prior to or shortly after infection with D-1 at 37° C, increases both the proportion of cells surviving infection and the frequency of lysogenization among the survivors. The increase, in both instances, is approximately twofold. Bertani (3) reported that chloramphenicol treatment increased the lysogenic response to phages P-2 and P-22. In addition, Christensen (4) obtained similar results with phage P-1 and subsequently reported (5) that chloramphenicol decreased the time required for lysogeny to become established in potentially lysogenic cells. This latter observation might explain the increased frequency of lysogenization among survivors in the presence of chloramphenicol at 37° C in the D-1–O26(2) system.

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DEGRADATION OF RUTIN BY ASPERGILLUS FLAVUS. PURIFICATION AND CHARACTERIZATION OF RUTINASE[1]

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Abstract

Aspergillus flavus produces an adaptive glycosidase (rutinase) that hydrolyzes rutin to quercetin and rutinose. Production of rutinase occurs when the mold is grown on the glycosides rutin, hyperosid, and naringin, and on the aglycones quercetin, kaempferol, rhamnetin, 2,4-dihydroxybenzoic acid, and 3,4-dihydroxyphenylacetic acid, but not when grown on glucose, galactose, rhamnose, or rutinose. Rutinase, after partial purification, is relatively stable when stored at −20° C, and is most stable and most active at pH 5.6. The enzyme is quite specific, hydrolyzing the 5-glucoside of sakuranetin, the 3-rutinoside and 3-galactoside of quercetin, but not the 3-L-rhamnoside nor any of the common glycosides. The hydrolysis of rutin is carried to completion aided by the insolubility of the aglycone quercetin in water.

Introduction

A search for microorganisms capable of attacking rutin revealed that several strains of *Aspergillus flavus* possessed this property (29). *A. flavus* (PRL 1805) elaborated extracellular enzymes which degraded rutin to carbon monoxide (22), rutinose [6-O-(6-deoxy-α-L-mannopyranosyl)-D-glucose] (2), phloroglucinol carboxylic acid, and protocatechuic acid (29). The first of the degradative reactions is the cleavage by "rutinase" of the glycosidic bond to yield quercetin and rutinose. The second step, or steps, in the degradative reactions is the oxidative cleavage by "quercetinase" of the heterocyclic ring with the production of carbon monoxide and a depside (29). This paper describes the purification and characterization of the rutinase produced by *A. flavus* when grown on rutin.

Materials and Methods

Chemical

A commercial preparation of rutin (Mann Research Laboratories) was purified by crystallization from absolute ethanol as the alcoholate and then recrystallization as rutin trihydrate from hot water (3). The ratio of the absorbancy of an alcoholic solution of rutin trihydrate at 375 mμ to that at 362.5 mμ was 0.877. Pure rutin has a ratio of 0.875 ± 0.004 (3).

DEAE cellulose was prepared for use by suspending 50 g in 4 liters of 0.02 *M* barbital (5,5-diethylbarbituric acid) and removing the fines by decantation. The procedure was repeated until the pH of the supernatant was 5.6 to 5.8. The DEAE cellulose was collected on a fritted glass Büchner funnel and then dried at room temperature.

Microbiological

Aspergillus flavus (PRL 1805) was maintained on agar slants containing

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0.1% rutin, 2% potato dextrose agar (Difco), and 1% agar. The extracellular enzymes were produced in 25 ml of medium in 250-ml Erlenmeyer flasks, or in 20 liters of medium in stainless steel fermentors, as previously described (28). Occasionally, as in the testing of substrates for their ability to induce the production of rutinase, 10 ml of medium was fermented in 50-ml Erlenmeyer flasks. The substrate and basal medium were sterilized separately, aseptically mixed, inoculated, and incubated at 30° C. The flasks were agitated on a rotary shaker while the fermentors were aerated at 5 liters of air per minute and stirred at 350 r.p.m. Cultures were harvested after 55 to 60 hours' incubation, and the mycelium removed by filtration; using Millipore filters (pore size 0.45 μ diameter) for small volumes and Whatman No. 3 paper for large volumes. The filtrates had a pH of 6.2–6.6 and when necessary were stored at 2° C overnight.

Enzymatic

One unit of enzyme was defined as the quantity of enzyme which hydrolyzed 1 micromole of substrate per hour under standard conditions. Two methods of assay were employed, a colorimetric procedure that measured the amount of rutinose released within 10 minutes and a spectrophotometric method that enzymatically measured the rate of formation of quercetin (Fig. 1).

In the first method, a portion of the enzyme preparation containing 0.2–3.5 units of rutinase was diluted to 1.0 ml with 0.1 *M* succinate buffer, pH 5.6, and incubated at 30° C. The reaction was begun by the rapid addition of 1 mg of rutin in 4 ml of 0.1 *M* succinate buffer, pH 5.6, using a Chaney pipette (16). (The saturated solution of rutin was prepared daily by dissolving the substrate in hot succinate buffer, then cooling the solution to room temperature and adjusting the volume.) The reaction was stopped after 10 minutes by heating the mixture in a boiling water bath for 5 minutes. The reaction mixture was poured into a 12-ml conical centrifuge tube containing 400 mg Magnesol to adsorb the flavonoid components (27). The Magnesol was suspended by stirring and removed by centrifugation. A 2.0-ml aliquot of the clear, colorless supernatant was transferred to a colorimeter tube containing 1.0 ml water, then 6.0 ml anthrone reagent was added rapidly from a Chaney pipette. The solution

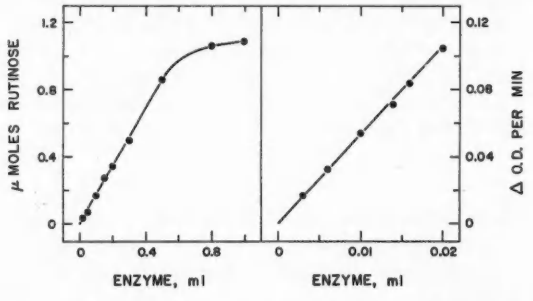

FIG. 1. (*a*) The relationship between concentration of rutinase and the amount of rutinose released in 10 minutes at 30° C. (*b*) The relationship between the concentration of rutinase and the loss in absorbance at 359 mμ in the presence of excess quercetinase.

was shaken vigorously and allowed to stand at room temperature for 15–20 minutes before reading at 540 mμ in an Evelyn photoelectric colorimeter (15). When the reaction mixture contained substances that interfered with the anthrone reagents, and that could not be quantitatively removed by Magnesol, reducing sugar was measured by the Somogyi–Nelson colorimetric assay (16, 17, 23).

The rapid spectrophotometric method was used after quercetinase free of rutinase had been obtained. This method is based on the loss in absorbancy resulting from the oxidative cleavage of the heterocyclic ring by quercetinase. The single addition of rutinase or of quercetinase to the solution of rutin had no effect on absorbance, but the addition of both enzymes resulted in a disappearance in absorbance at 357 and 370 mμ. Rutin in the reaction mixture exhibited maximal absorption at 357 mμ and quercetin at 370 mμ as compared to published values of 362 and 375 mμ for rutin and quercetin dissolved in ethanol (3, 9). At 359 mμ the molar extinction coefficients of both compounds were equal (where l = 1 cm, C = 10 μmoles per liter, O.D. = 0.163).

The glycosidase activity was determined at room temperature by adding to a cuvette (l = 1 cm) 0.7 ml of substrate, 0.7 units of quercetinase, water, and from 0.07 to 0.27 units of rutinase to a final volume of 1 ml. Readings were taken at 359 mμ at 1-minute intervals for 5 or more minutes. The units of enzyme were then equal to the average rate of change in optical density per minute × 60 × 0.0614. The substrate was prepared by dissolving 12.5 mg of rutin in 100 ml of hot water, cooling, then adding 100 ml of succinate buffer (2.1 g of succinic acid per 100 ml adjusted to pH 5.6 with sodium hydroxide) and adjusting the volume to 250 ml. This substrate was fairly stable and could be kept at room temperature for 2 or 3 days.

Quercetinase was determined in a similar fashion with the cuvette containing 0.7 ml of rutin, 0.7 units of glycosidase, water, and suitably diluted quercetinase to give a volume of 1 ml.

Analytical

Protein was determined by the colorimetric method of Lowry *et al.* (13) and rhamnose by the method of Dische and Shettles (1).

Experimental

Induction of Rutinase

The ability of a number of compounds to induce production of the glycosidase was determined by growing *Aspergillus flavus* on glucose for 48 hours, aseptically homogenizing the mycelium in a Waring blendor, and using this to inoculate a series of 50-ml Erlenmeyer flasks containing various substrates in 10 ml of medium. After incubation for 72 hours on a rotary shaker at 30° C, the mycelia were used to inoculate a second series of flasks containing the respective substrates.

The filtrates from both series of flasks were assayed for rutinase and since those substrates that induced production of the enzyme did so equally well in both series, the results were averaged (Table I).

In an earlier paper (29), it was postulated that production of rutinase was induced by the glycoside or the aglycone but not by the free sugars. The

TABLE I

Ability of various substances to induce production of rutinase

Substrate	Rutinase, units/ml	Substrate	Rutinase, units/ml
Rutin	9.5	2,4-Dihydroxybenzoic acid	1.5
Hyperosid	8.6	3,4-Dihydroxyphenylacetic acid	1.8
Kaempferol	4.9	D-Glucose	0
Rhamnetin	3.1	D-Galactose	0
Quercetin	2.0	Rutinose	0
Quercetin and rutinose	2.9	Naringenin	0
Naringin	2.2	None*	0

*Basal medium: 0.002% glucose, 0.3% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.8% $(NH_4)_2SO_4$, and 0.1% $MgSO_4$.

NOTE: The following did not induce production of rutinase: D-catechin, chalcone, fisetin, flavone, hesperidin, 3-hydroxyflavone, morin, neohesperidin, robinetin, taxifolin, amygdalin, esculin, helicin, khellin, phloridzin, salicin, saligenin, salicylic acid, cellulose, cellobiose, maltose, rhamnose, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, phenyl β-D-glucopyranoside, phloroglucinol, protocatechuic acid, 3,5-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid.

presence of rutinase in filtrates from *A. flavus* grown on quercetin, rhamnetin, and kaempferol supports this hypothesis (Table I). These three flavonols differ only in the groups on carbons 7 and 3′ (10). Naringin, the 7-neohesperidoside of naringenin, a flavanone (4), has quite a different structure, but does induce production of some rutinase. Naringenin, the aglycone, did not. This might be explained if naringin were contaminated with flavonols or flavonol glycosides, but none could be detected by paper chromatography.

Purification of Rutinase

The culture filtrates contained from 0.2 to 0.5 mg of protein per milliliter. Attempts to increase the concentration of protein to more workable levels by adsorption and elution from various materials failed because of the high salt content of the filtrates and because those adsorbents that were effective bound the enzyme so tightly that elution was incomplete. The enzyme could be concentrated by foaming (12), but this was abandoned as approximately 30% remained in the mother liquors. Small volumes could be conveniently concentrated by lyophilization with quantitative recovery of the enzymes. Large volumes were concentrated in a Mojonnier Lo-Temp evaporator at temperatures below 25° C. Since the pH dropped during evaporation, 0.1 g of sodium succinate was added for each liter of filtrate to prevent the pH from falling below 5.6. Under these conditions, about 10 to 20% of the enzymes was lost (Table II). The concentrated preparations were then used immediately or stored at −20° C at pH 5.6.

The concentrate (9.7 mg protein per ml) from 54.8 liters of filtrate was treated with DEAE cellulose to remove insoluble debris and colored materials. Fifteen grams of previously equilibrated and dried DEAE cellulose were suspended in 300 ml of 0.02 *M* barbital and added slowly with stirring to the concentrated filtrate (2000 ml, pH 5.6) and mixed for 5 minutes. The mixture was filtered through a coarse fritted glass funnel, and the pad of DEAE cellulose rinsed with a little 0.02 *M* barbital and sucked dry.

The clarified filtrate was adjusted to pH 6.0 and sufficient ammonium sulphate added to give 90% saturation. The brown precipitate was recovered by

centrifugation at 25,000 × *g* with a flow rate of approximately 20 ml per minute in a Servall SS-3 equipped with a Szent-Gyorgyi Blum continuous-flow attachment. The brown precipitate was suspended in 30 ml of 0.002 *M* succinate buffer, pH 5.6. The insoluble material was separated by centrifugation and extracted twice more with 20- to 30-ml portions of 0.002 *M* succinate buffer.

TABLE II

Purification of glycosidase

Step	Volume, ml	Rutinase			Quercetinase
		Units × 10^{-3}	Purification*	% recovered	Units × 10^{-3}
Filtrate	54,800	630	1	100	520
Concentrate	2,000	498	1	79	480
Neg. DEAE	2,350	488	1	77	450
$(NH_4)_2SO_4$ 0–90	100	366	8	58	250
Ethanol	30	350	14	55	250
Dialysis	50	312	13	50	147
DEAE	75	280	51	44	Trace
Neg. gel	95	280	57	44	0
$(NH_4)_2SO_4$ 65–77	8	160	113	25	0

*Purification is equal to the specific activity of the fraction divided by the specific activity of the filtrate where specific activity equals units per milligram of protein.

The combined extracts (100 ml, pH 5.6) were cooled to 0° C and 300 ml cold (−30° C) ethanol added with stirring. A voluminous white precipitate, recovered by centrifugation at 10,000 × g for 1 minute, was suspended in a minimal volume of 0.002 *M* succinate buffer, pH 5.6, and dialyzed overnight against cold flowing 0.002 *M* succinate buffer, pH 5.6. The dialyzed solution was made 0.02 *M* with respect to barbital, adjusted to pH 5.6, and added to barbital-equilibrated DEAE cellulose (12 g) in a column 2.5 cm × 24 cm. The column was then rinsed successively with 20 ml 0.02 *M* barbital and 60 ml of 0.02 *M* succinate – 0.02 *M* barbital buffer, pH 5.6. The rutinase was removed from the adsorbent by exponential gradient elution* with 0–0.4 *M* NaCl in 0.02 *M* succinate–barbital buffer, pH 5.6. The eluate flowed from the column at approximately 3 ml per minute and was collected in 10-ml fractions.

During elution, the pH rose to approximately 9.0 and then fell to below 6.0. Rutinase usually appeared as the pH declined. Quercetinase was eluted at the highest concentrations of NaCl. It was preferable, however, to stop the gradient elution when the pH dropped below 6.0, and elute the quercetinase with 0.5 *M* NaCl in 0.02 *M* succinate–barbital buffer, pH 5.6.

The tubes containing the rutinase, or quercetinase, were pooled. The preparation of quercetinase was relatively stable when stored at −20° C and was used in the spectrophotometric assay. Traces of rutinase could be removed by holding the preparation of quercetinase at 70° C, pH 6.0, for 20 minutes.

*The gradient elution apparatus was designed by Dr. J. E. Watkin of this laboratory. A gradient which is approximately exponential is obtained when the cross sectional area of the receiving cylinder is just under twice that of the supply cylinder. The supply cylinder contained 200 ml of 0.4 *M* NaCl in 0.02 *M* succinate–barbital buffer, pH 5.6, and was connected at the bottom to a mixing cylinder containing 400 ml of 0.02 *M* succinate–barbital buffer, pH 5.6.

The pooled eluates containing rutinase (75 ml) were adjusted to pH 6.0 with dilute NaOH and 2.19 g of sodium chloride was added. Calcium phosphate gel (15 ml; 12.8 mg calcium phosphate per ml) was then added dropwise, with stirring, and mixed for 5 minutes. The gel was removed by centrifugation and discarded. This treatment removed the last traces of quercetinase activity.

The supernatant was adjusted to pH 6.0 and diluted to 100 ml. Ammonium sulphate (40 g, 65% saturation) was added, and the solution, then at 2° C, allowed to stand at room temperature for 15 minutes before the precipitate was removed by centrifugation and discarded. Ammonium sulphate (9.5 g, 77% saturation) was added to the supernatant and the precipitate collected as before. The second precipitate was suspended in 5.0 ml of 0.1 *M* succinate buffer, pH 5.6, and filtered through glass paper. The results of this purification procedure have been summarized in Table II.

Stability

Rutinase in both the culture filtrate and the concentrated filtrate is quite stable for a week at 0° C at pH 5.6. About 10% of the activity is lost under such conditions and even less when stored at −20° C. After 3 weeks at 0° C about 70% of the activity remains and at −20° C, about 85%. The purified preparation retained 75% of its original activity after storage for 2 weeks at −20° C. Frequent freezing and thawing was detrimental, resulting in a 40 to 50% loss in the same period. The addition of 0.1% ammonium sulphate and succinate to a dialyzed preparation had some protective effect when the preparations were held at 55° C for 10 minutes. However, rutin and quercetin labilized the enzyme resulting in a loss 20% greater than the control. Glucose, rutinose, thiogel, glutathione, glycine, and glycylglycine had no effect.

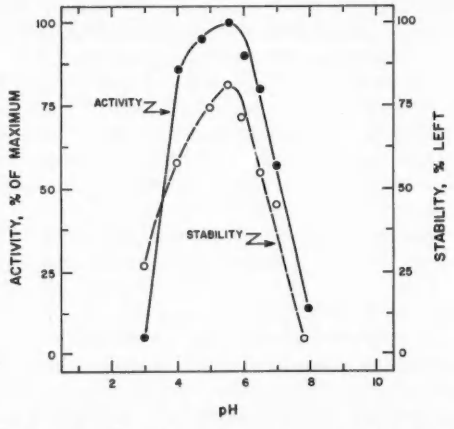

FIG. 2. Relationship of activity and stability to pH. The activity at different pH values was determined by the colorimetric method using 0.1 *M* succinate–phosphate buffers. The stability was determined by holding the enzyme in 0.1 *M* succinate–phosphate buffers at 55° C for 10 minutes. The enzyme was then diluted in cold 0.1 *M* succinate buffer, pH 5.6, and the activity determined by the colorimetric procedure.

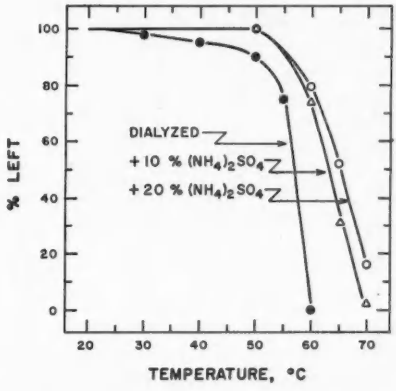

FIG. 3. Stability of rutinase. The enzyme (13 units) in 0.1 *M* succinate buffer, pH 5.6, (volume 1.0 ml) was held at the desired temperature for 20 minutes, cooled quickly, and assayed by the spectrophotometric method.

Rutinase is most stable and most active at pH 5.6 (Fig. 2). This is higher than the optimum pH of 3.5 to 4.5 found for a commercial glycosidase that attacks naringin (24, 25). At pH 5.6, the enzyme is relatively stable for 20 minutes up to 50° C (Fig. 3). At higher temperatures the enzyme becomes increasingly thermolabile. Ammonium sulphate increased the stability of the enzyme.

Inhibition and Activation

The ability of a large number of inorganic and organic compounds to inhibit or activate rutinase was tested by preincubating for 15 minutes at 20° C a solution (1.0 ml) containing 8 units of the enzyme, 50 μmoles of succinate buffer (pH 5.6), and the test substance at the desired concentration. A portion of this was then assayed for activity by the colorimetric procedure. Controls employing enzyme denatured by heat were incubated concurrently with each inhibitor.

Few of the inorganic or organic compounds tested had any effect on rutinase. Versene, $KH(IO_3)_2$, and $K_3Fe(CN)_6$ (0.01 *M*) were slightly stimulatory. At 0.02 *M* or less, the acetate, nitrate, or chloride salts of lead, cobalt, zinc, copper, barium, and iron were innocuous. Likewise sodium fluoride, fluoroacetate, sulphide, sulphite, azide, arsenate, and cyanide at 0.02 *M* did not inhibit nor did a number of sulphhydryl reagents. Aluminum was the most effective inorganic inhibitor (Table III), and could be inhibiting either by acting on the enzyme or by chelating with the substrate (3, 5). The most effective organic inhibitors other than formaldehyde were the nitrogenous bases: pyridine, dimethylamine, and quinidine. However, tris(hydroxymethyl)-methylamine, triethanolamine, ethylenediamine, hydroxyquinoline, and urea were not inhibitory at concentrations of 0.01 *M*, nor was quercetin at 0.0001 *M* and rutinose at 0.01 *M*.

TABLE III

Inhibition of rutinase

Compound	Concentration, *M*	Inhibition, %
Aluminum chloride	0.02	69
Mercuric chloride	0.02	58
Potassium iodate	0.005	25
Silver nitrate	0.02	18
Formaldehyde	0.005	30
Pyridine	0.01	97
Dimethylamine	0.02	84
Quinidine	0.002	16

Specificity

The ability of the purified rutinase preparation to hydrolyze various glycosides was first tested with the colorimetric procedure using the Somogyi–Nelson (17, 23) method for detecting the sugar released by 8 units of glycosidase acting on 1 mg of substrate for 10 minutes at 30° C. Rutin and hyperosid were attacked. There was no detectable activity on hesperidin, naringin, quercitrin, amygdalin, arbutin, esculin, helicin, *o*-nitrophenyl β-D-galactoside, phenyl β-D-glucoside, phloridzin, cellobiose, glycogen, maltose, melibiose, raffinose, rutinose, starch, sucrose, trehalose, methyl β-L-arabinoside, methyl α-D-glucoside, methyl β-D-glucoside, methyl β-maltoside, methyl α-D-mannoside, and methyl α-L-rhamnoside.

The specificity was again tested when additional flavonoid glycosides were available. In this survey, 500 units of glycosidase were incubated with 10 mg of substrate for 10 minutes at 30° C in 5 ml of 0.02 *M* succinate buffer, pH 5.6. Instead of the batchwise adsorption of the flavonoid components, they were removed by passing the reaction mixture through a 1-cm wide column of 800 mg of Magnesol and the free sugar rinsed through into a volumetric flask (25 ml) with 0.1 *M* NaCl. The sugar content of this eluate was determined (1, 15). Under these conditions, rutin, hyperosid, and sakuranin were hydrolyzed and, to the extent of 5% or less, hesperidin, neohesperidin, naringin, and quercitrin. Rutinase thus appears to be quite specific.

The effect of the concentration of rutin on the rate of the reaction is shown in Fig. 4. The graphical method of Lineweaver and Burk (11, 14) was used to estimate the enzyme dissociation constant. K_s for rutin was calculated to be 7.3×10^{-5} moles per liter.

Stoichiometry

The stoichiometry of the reaction was estimated by incubating at 30° C 5-ml portions of a solution of rutin (1.76 μmoles) with 15 units of glycosidase in a series of tubes, then at varying intervals stopping the reaction and determining the sugar released colorimetrically, the rutin and quercetin spectrophotometrically (21).

Inorganic salts were found to interfere with the spectrophotometric determination of rutin and quercetin by shifting the spectrum. For this reason, no buffer was employed in the reaction mixture other than that added with the enzyme. Even without buffer, the pH remained steady at 5.5. The reaction

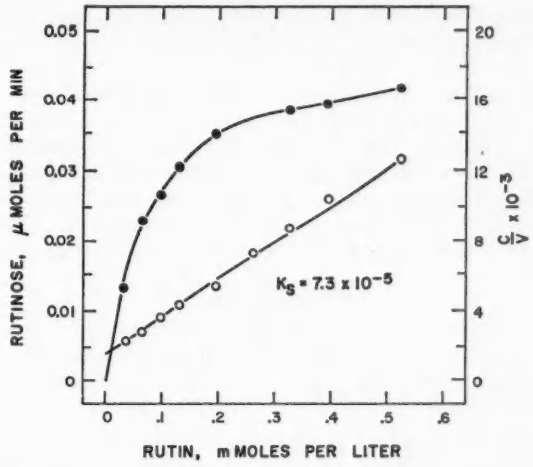

FIG. 4. Effect of the concentration of rutin upon the rate of hydrolysis. The reaction mixture (5.0 ml) contained rutin, 3.5 units of glycosidase, and 500 μmoles of sodium succinate buffer, pH 5.6.

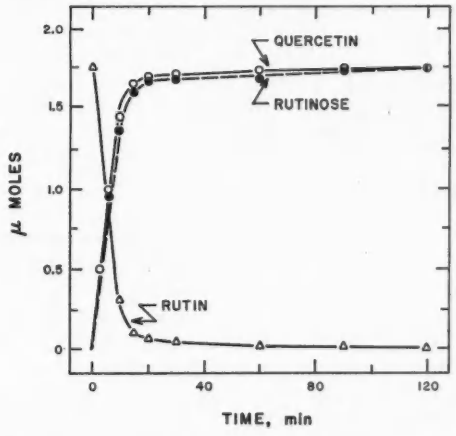

FIG. 5. The hydrolysis of rutin by rutinase. The reaction mixture contained 1.76 μmoles of rutin and 15 units of rutinase.

was stopped by adding 20 ml of acidified ethanol (1 ml of 0.2 *N* acetic acid per liter) at room temperature. Portions of this were then diluted fivefold in acidified ethanol and the optical density determined in the Beckman spectrophotometer (21).

The reaction in the tubes used for the determination of rutinose was stopped by adding 0.1 ml of 1 *N* H_2SO_4 and, after neutralization, the remaining rutin

and quercetin were removed by passing the reaction mixture through a column 1 cm wide containing 800 mg of Magnesol. The quercetin and rutin were absorbed and the rutinose was rinsed through into a 25-ml volumetric with 0.1 *M* sodium chloride. An aliquot of this filtrate was used to determine rutinose (1, 16).

The reaction was 5% completed after 6 minutes, 95% completed after 20 minutes, and 99.5% completed after 120 minutes. The reaction, as seen in Fig. 5, goes to completion under the conditions employed as quercetin is practically insoluble in water. A precipitate began to form 10 to 15 minutes after addition of the enzyme, but this readily dissolved in ethanol.

Discussion

The glycosidase produced by *Aspergillus flavus* when grown on rutin is, like most induced enzymes, quite specific. Rutinase does not attack any of the common glycosides and appears to be restricted to hydrolyzing certain flavonoid glycosides, those containing rutinose, glucose, or galactose. The results obtained agree with the specificities generally attributed to β-D-glucosidases with the addition of a marked specificity for the aglycone (6, 7, 8, 19). β-Glucosidases exhibit specific requirements for the configurations at carbons 1, 2, and 3 of the sugar moiety and also a degree of specificity for carbons 4 and 5, but little for carbon 6 (20, 26). This generalization may also apply to rutinase since it hydrolyzes the 5-glucoside of sakuranetin (sakuranin) and the 3-rutinoside and 3-galactoside of quercetin, but not the 3-L-rhamnoside of quercetin. Although the bond between rutinose and quercetin in rutin has not been established, there is some indirect evidence that it may be a β-glycosidic bond. For example, Nystrom *et al.* (18) have shown that the 3-glucoside and the 7-glucoside of quercetin, like the 7-glucoside of hesperetin, were hydrolyzed by emulsin but not by an α-glycosidase. The 7-glucoside of hesperetin was prepared from hesperidin by partial hydrolysis with formic acid. Zemplén and Tettamanti (30) established that hesperidin is hesperetin 7-β-rutinoside. Since rutinose in this glycoside and in rutin are attached to the aglycone through glucose, one might assume on the basis of the results obtained by Nystrom *et al.* that rutin is quercetin 3-β-rutinoside. If so, then hyperosid and sakuranin, which are attacked by rutinase, are β-glycosides. Rutin, however, is not attacked by emulsin, perhaps because of steric hindrance from the L-rhamnose attached to carbon-6 of D-glucose (26).

Rutinase is a relatively stable enzyme little affected by most inorganic salts. The enzyme can withstand 50° C for short periods of time and resists surface denaturation. Rutinase is, however, sensitive to pH, and is rapidly inactivated below pH 4.0 and above pH 6.5. These properties are similar to those reported by Jermyn for the cellulases of *A. oryzae* (5, 6), except that rutinase is less affected by such compounds as sulphide, cyanide, and 8-hydroxyquinoline. Like other glycosidases previously studied, rutinase is not activated by any inorganic salts and compared to quercetinase is relatively stable to prolonged dialysis.

The data in this paper support the previous hypothesis (22, 28, 29) that hydrolysis of rutin by a glycosidase is the first step in the series of reactions

involved in the degradation of rutin. The spectrophotometric assay developed for rutinase depends on the inability of quercetinase to break the heterocyclic ring unless rutinose is first removed from rutin.

When the work on the degradation of rutin began, we believed our strain of *A. flavus* did not produce an extracellular enzyme that hydrolyzed rutinose, and culture filtrates were conveniently used for preparing rutinose. Subsequently, particularly after the adoption of the synthetic medium (28), the presence of an enzyme that hydrolyzed rutinose was detected in some culture filtrates, but in low concentration. Thus *A. flavus* likely hydrolyzes rutinose to glucose and rhamnose before further metabolizing the sugars.

Acknowledgment

Mr. K. L. Phillips generously assisted in concentrating the enzyme and Miss C. van Loenan and Mr. A. S. Sieben were the technical assistants. The authors wish to thank them, and also to express their appreciation for the advice and help received from Dr. A. S. Perlin and Dr. G. Talbot.

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A NEW VIRULENCE TEST FOR STAPHYLOCOCCUS AUREUS AND ITS APPLICATION TO ENCAPSULATED STRAINS[1]

BILL B. WILEY

Abstract

An encapsulated variant of *Staphylococcus aureus* was obtained after laboratory passage in Casamino Acids glycerol broth. Hyperimmune rabbit or rooster serum, prepared by injection of washed, formalin-killed, whole cell vaccines, regularly produced specific capsular reactions* of 4-to 8-hour broth cultures of homologous organisms. Intravenous injection of the encapsulated variant into 12- to 13-day-old embryonated hens' eggs regularly produced death when 1000–2000 organisms were injected. The passive protective properties of hyperimmune serum or human gamma globulin were readily demonstrated if 5–20 LD_{50} doses of a young broth culture of the encapsulated variant were mixed with the antiserum prior to injection into the eggs. The finding of anticapsular antibodies in samples of human plasma indicated that the capsular antigen or one closely related to it was produced in vivo.

Introduction

The study of immunity in staphylococcal infections has had advocates from two schools of thought: those inclined to the view that antitoxin plays a dominant role in staphylococcal lesions and those who felt that immunity was chiefly antibacterial. Present thinking relegates antitoxic immunity to a smaller role and leaves the question of other humoral factors in host resistance open to discussion. Lyons (14) reported finding encapsulated staphylococci in pus and blood cultures in 1937. Although he was unable to demonstrate a specific capsular reaction with his strains, he contended they were encapsulated on the basis of a capsule stain he devised. He found 3-hour cultures of his staphylococcal strains to be resistant to phagocytosis and ascribed this resistance to the presence of a capsule.

About the same time Forssman (8) found rabbits to be resistant to challenge by living staphylococci following immunization with formalinized vaccines, and as he was unable to detect significant levels of staphylococcal antitoxin in the serum of the rabbits, he concluded the resistance was due to antibacterial factors.

The description of an encapsulated variant of *Staphylococcus aureus* in 1954 by Price and Kneeland (16) stimulated interest in the whole subject of immunity in staphylococcal infections. These workers were able to produce specific capsular reactions with their strain using a hyperimmune rabbit serum, but failed to find any difference in virulence between the parent strain and the encapsulated variant. The virulence tests were carried out in mice injected intraperitoneally with organisms suspended in 5% hog gastric mucin. Further work by Price and Kneeland (17) in 1956 revealed that many

*The term "specific capsular reaction" is used instead of "capsular swelling reaction" in accordance with the evidence summarized by Tomcsik (21).

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coagulase positive strains of *S. aureus* could produce capsular substances demonstrable by the specific capsular reaction. They concluded that no type specificity of the capsular substance was involved because 32 of 39 strains from various lesions underwent specific capsular reactions when a single antiserum was employed. My interest in the problem of virulence factors and host defenses against staphylococcal disease was stimulated by the work of Price and Kneeland (16, 17). I decided to attempt the isolation of mucoid variants of *S. aureus* and to study their virulence in a suitable host. A study of the passive protective properties of an antiserum containing anticapsular antibodies in high titer also seemed relevant to the problem.

Materials and Methods

Strains of S. aureus

Strains of *S. aureus* were obtained from the University Hospital, Saskatoon, after minimal subcultivation in Difco brain–heart infusion broth. Coagulase tests were carried out using 0.5 ml of an overnight culture of the strain in buffered nutrient glycerol broth and 0.5 ml of suitable human plasma. The tests were read after cultures were incubated 3 hours at 37° C in a water bath. Each strain was also tested for ability to ferment glucose and mannitol anaerobically, a characteristic which, according to Bergey (2), is important in defining the species *S. aureus*. The incubation temperature for all culturing, excepting eggs, was 37° C.

Media

For a passage medium a semisynthetic medium was devised. It contained the following ingredients: Casamino Acids, Technical (Difco), 8.60 g; phenol red, 0.010 g; potassium monohydrogen phosphate (anhydrous), 1.63 g; potassium dihydrogen phosphate, 0.454 g; glycerol, 20 ml; 3 *N* NaOH, 2.0 ml; and distilled water to make 1000 ml. These ingredients were autoclaved at 121° C for 20 minutes and to 1 liter of the sterile medium 10 ml of a 2% solution of magnesium sulphate and the following vitamins were added aseptically: thiamine, 10 μg, pyridoxine 1 mg, and nicotinic acid 200 μg. The pH of the medium should be 7.2 to 7.4. To prepare a solid medium of the above composition, agar was added to a concentration of 2%.

For specific capsular reactions, virulence studies, and plate counts, a nutrient glycerol broth was used. Its composition was as follows: Difco nutrient broth, 8.0 g; glycerol, 20 ml; sodium monohydrogen phosphate (anhydrous), 1.62 g; potassium dihydrogen phosphate (anhydrous) 0.24 g; distilled water to make 1000 ml. The final pH of the broth should be 7.4. The medium was sterilized at 121° C for 20 minutes. When a solid plating medium was desired, 2% of Difco agar was added to the broth. Except when a specific medium is mentioned, reference to broth or agar will imply use of buffered nutrient glycerol medium.

Vaccines and Immunization

Organisms for vaccine preparation were grown in the semisynthetic broth for 18 hours and killed by the addition of formalin to a final concentration of 0.5%. After incubation to insure killing of the organisms, they were washed

once with sterile buffered saline before injection. The final concentration of organisms was approximately one billion organisms per milliliter as determined by comparison with a McFarland nephelometer (15). White New Zealand rabbits or New Hampshire Red roosters received intravenous injections on 4 consecutive days each week. The initial dose was 0.1 ml and this was increased by 0.1 ml at each injection up to a maximum of 1.0 ml, except that the dose attained at week's end was repeated on the first day of each new week. This program, followed for 1 month, constituted one course of injections. Three such courses were administered before trial bleedings were made. This method of preparing antiserum was first reported by Alexander (1). A satisfactory antiserum produced specific capsular reactions when diluted 1:400. Antiserum, obtained after fasting the animals, was stored in the lyophilized state.

Virulence Test

Virulence tests were conducted in 13-day-old embryonated hens' eggs. Cultures of *S. aureus* were grown in broth for 8–12 hours. These were used to make 24-hour plate cultures from which inoculum for the tests was obtained. A large sweep of the growth was harvested from the plate by means of a sterile glass rod. This mass of growth was emulsified in 0.2 ml of broth. The final emulsification was made with a 3-ml Luer-Lok syringe fitted with a 20-gauge needle. One milliliter of broth was drawn into the syringe along with the 0.2 ml of partially emulsified staphylococci. This mixture was repeatedly drawn into the syringe and rapidly expelled into a sterile test tube fitted with a rubber vaccine cap. This inoculum was adjusted to an optical density difference of 0.38, as compared with broth, at 515 mμ in a Bausch and Lomb Spectronic "20" spectrophotometer. To 20 ml of broth in a screw-capped test tube with glass beads was added 2 ml of the inoculum and the tube shaken 100 times. At the end of the incubation period the tube was shaken again 100 times before the optical density was read. If the optical density difference was more than 0.38, the culture was diluted with sterile broth to a difference of 0.38. If the optical density difference was less than 0.38, the culture was used as it was. The culture was then diluted 1:500, 1:5000, 1:50,000 for injection and to 1:1,000,000 for the preparation of plates in sextuplicate for viable cell counts.

The eggs were candled to locate a large vein in the chorioallantoic membrane. The area was marked and a window cut through the shell over the vein. The window was lifted off without disturbing the underlying shell membrane and a drop of sterile mineral oil placed on the shell membrane to make it transparent. Using a 0.25-ml tuberculin syringe fitted with a 27-gauge needle, the inoculum of 0.05 ml was injected intravenously. The window and hole were closed with melted paraffin and the eggs incubated at 37.8° C in a Jamesway egg incubator operated at a relative humidity of 62%.

Passive Protection Test

Essentially the passive protection tests were carried out in the same way as the virulence test. An LD_{50} determination was always carried out in conjunction with the passive protection test. The passive protective properties of antiserum were measured by mixing the staphylococci with the antiserum and

allowing the mixtures to stand at 0–6° C for 3 hours before injection. These tests were conducted using the encapsulated strain of staphylococcus diluted 1:500. The organisms were first diluted 1:100 in broth, then the final dilution made in antiserum by mixing 1 part of staphylococci 1:100 with 4 parts of antiserum. The first dilution of the LD_{50} determination was 1:500 and this dilution usually killed 100% of the eggs. All antisera were inactivated at 56° C for 30 minutes prior to use in the protection test.

Specific Capsular Reaction Test

Tests for specific capsular reactions were carried out using 6–8 hour cultures of the organisms in 3-ml quantities of broth. A single colony from a 24-hour plate culture of the strain being tested was inoculated into broth and incubated 6–8 hours. To 3 loopsful of antiserum was added 1 loopful of this broth culture and 1 loopful of aqueous methylene blue. The antiserum, organisms, and dye were mixed on a vaseline-rimmed coverslip and inverted on a microscope slide. These slides were incubated overnight before being read because the reaction was stronger and more easily discernible after such incubation.

Isolation of Encapsulated Variants

Bigger, Boland, and O'Meara (4) had shown that cultivation of *S. aureus* in a medium containing fermentable carbohydrate led to the emergence of mucoid variants if the cultures were simply left in the acidic environment for several months after inoculation. We used the Casamino Acids glycerol broth for this purpose. One month after inoculation the cultures were streaked on Casamino Acids glycerol agar plates and the colonies tested for mucosity by means of a straight inoculation needle. Experience with the mucoid variant supplied by Price and Kneeland (16) enabled us to recognize the highly mucoid colonies resembling their mucoid variant. When a mucoid colony appeared it was subcultured on Casamino Acids glycerol agar plates until a pure culture was obtained.

Results

By means of laboratory passage in Casamino Acids broth according to the technique of Bigger, Boland, and O'Meara (4), five strains of mucoid encapsulated *S. aureus* were isolated. In some instances the mucoid variants appeared only after cultivation of the strains and their storage in the acid culture environment for 1 or 2 months. The first mucoid variant obtained was that from an infected suture. The colonies of this strain were gummy and lifted off the agar as a single mass when touched with an inoculating needle. They adhered to the needle so tenaciously that it was difficult to shake them off and plant them in broth tubes. This mucoid variant, designated the "wound" strain, was employed to immunize rabbits and roosters for antiserum production. At least 3 monthly courses of immunization were necessary to produce antisera possessing a high level of antibody. The rabbit antiserum possessed the highest titer, but the rooster antiserum reacted more rapidly and made the capsules appear larger. Figure 1 is a photomicrograph of the specific capsular reaction of the wound staphylococcus in the presence of rooster antiserum. Figure 2 is a photomicrograph of the specific capsular

PLATE I

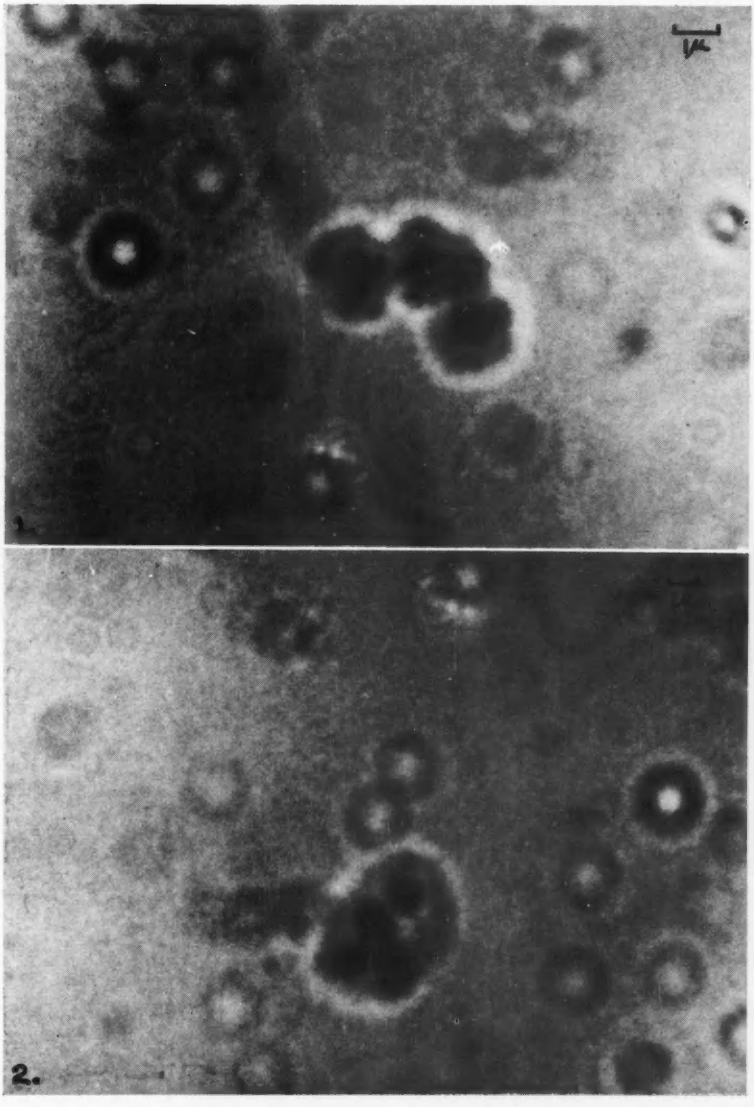

FIG. 1. Specific capsular reaction of the wound staphylococcus in the presence of rooster antiserum.

FIG. 2. Specific capsular reaction of the wound staphylococcus in the presence of rooster antiserum showing organisms apparently sharing a capsule.

reaction of the wound staphylococcus in the presence of rooster antiserum showing a group of staphylococci sharing a capsule.

It seemed desirable to study the virulence of the mucoid variants by means of animal tests. Most animal hosts are quite resistant to staphylococcal infections. Rabbits (8) and mice (10) have both been used in the study of staphylococcal virulence. Rabbits have the disadvantage of expense and require large amounts of space if suitable numbers are used. Mice are highly resistant to infection by *S. aureus* even when the organisms are suspended in hog gastric mucin. Frappier and Sonea (9), and Frappier, Sonea, and Panisset (10) showed that the 19-day-old embryonated hen's egg was highly susceptible to subcutaneous inoculation of *S. aureus*. We were impressed by the sensitivity of this technique because the LD_{50} was only 10–100 organisms. The traumatic death rate, however, was rather high. The injection of 19-day-old eggs precluded a long observation before hatching occurred. Although Frappier *et al.* (9, 10) used failure to hatch as a criterion of fatal infection, our experience indicated that a certain percentage of these eggs failed to hatch whether or not they were injected. To measure the ability of the organism to resist phagocytosis in the embryonated egg, we decided to inject the organisms intravenously. Having found that 13-day-old eggs possessed veins of sufficient size, we injected 200 eggs with sterile broth to determine the traumatic death rate. It was found that this varied from 3 to 5%. It was also learned that traumatic deaths almost always took place within 18 hours, hence they could be discovered by candling the eggs 18 hours after injection. The LD_{50}'s of the encapsulated variants calculated by the probit transformation method (11) are given in Table I.

TABLE I

Virulence of encapsulated *Staphylococcus aureus* strains produced by laboratory passage

Strain*	Source	Log LD_{50}†	Phage pattern
Wound M	Infected suture	2.98 ± .52	52/52A/80
Wound M		3.08 ± .48	
Wound M		3.22 ± .22	
RLM	Egg passage of staphylococcal	3.24 ± .42	52/52A/80
RLM	pneumonia strain	3.47 ± .26	
RLM		3.66 ± .74	
204M	Septicemia	3.03 ± .48	7/47/53/54/75/77
204M		3.09 ± .56	
204M		3.52 ± .04	
203M	Lung post-mortem	3.54 ± .12	7/47/53/75/77/81
203M		3.64 ± .06	
203M		3.65 ± .44	
206M	Peritoneal fluid post-mortem	3.60 ± .72	3A/3B/3C/57/71
206M		3.88 ± .52	
206M		3.91 ± .14	

*Test made with 18-hour cultures.
†LD_{50}'s expressed as the logarithm to the base 10 of the number of organisms producing 50% death of the eggs. The 95% confidence limits based on a normal distribution are shown.

The LD_{50} is expressed as the logarithm to the base 10 of the number of organisms producing 50% death of the eggs. Triplicate tests were carried out at intervals of 1 week to assess the reproducibility of the test. The weekly variations in virulence of the same strain were not significant when tested by the "*t*" test. The egg virulence test thus appears to be reproducible. All strains tested reacted in the specific capsular reaction test with a single antiserum, that prepared against the wound mucoid strain. All were coagulase positive and fermented glucose and mannitol anaerobically, characteristics which, according to Bergey (2), are typical of *S. aureus*.

A similar study carried out with a group of *Staphylococcus epidermidis* strains is shown in Table II.

TABLE II

Virulence tests of *S. epidermidis* strains

Strain*	Source	Number of organisms injected†	Specific capsular reaction
3858	Ear	9,000,000	−
3961	Urine	5,000,000	+
Protz	Nose	2,000,000	−
White I	Skin	3,000,000	−
White II	Skin	5,000,000	−
3780	Throat	3,500,000	−

*Tests made with 18-hour cultures.
†In no instance did 50% of the eggs die.

The LD_{50}'s of these strains were indeterminable since none of them caused 50% death of the eggs in any dilution tested. All were coagulase negative and failed to ferment mannitol anaerobically. Only strain 3961 gave a positive specific capsular reaction test. The significance of this finding is commented upon in the discussion.

The virulence of the encapsulated *S. aureus* strains suggested that the capsule might play a role in the virulence of the organism. Non-encapsulated strains of *S. aureus* with which to compare the encapsulated variants were not available. It was very difficult to obtain cultures in which none of the organisms exhibited a capsule. It was hypothesized that if the capsule played a role in the virulence of the organism, then antibodies to the capsule might exert a protective effect on the egg. Pure anticapsular serum was not available since our antiserum was prepared against formalinized whole cells but the antiserum did possess a high titer of anticapsular antibodies as measured by the specific capsular reaction test. Antisera eliciting a specific capsular reaction when diluted 1:400 were usually used. Passive protection tests were carried out using rabbit immune serum and rabbit and human gamma globulin fractions. Control tests employing normal rabbit and rooster serum were also carried out. The results of the protection tests are shown in Table III.

With but one exception the antiserum or globulin protected the eggs against doses of living staphylococci ranging from 5–20 LD_{50}'s. The actual amount of serum or globulin injected with the organisms was 0.04 ml of undiluted

TABLE III

Passive protection against the encapsulated wound staphylococcus

Material	Challenge LD_{50}'s*	Protection as % survival†	Mean % survival
Hyperimmune rabbit serum	7	87	
Hyperimmune rabbit serum	10	72	86
Hyperimmune rabbit serum	10	100	
Hyperimmune rooster serum	5	60	
Hyperimmune rooster serum	8	53	53
Hyperimmune rooster serum	10	46	
Immune rabbit globulin	10	81	
Immune rabbit globulin	10	75	79
Immune rabbit globulin	10	80	
Normal human globulin	5	93	
Normal human globulin	9	80	82
Normal human globulin	10	73	
Normal rabbit serum	10	13	
Normal rabbit serum	10	22	19
Normal rabbit serum	10	22	
Absorbed normal rabbit serum	10	0	
Absorbed normal rabbit serum	15	0	
Absorbed normal rabbit serum	20	0	
Normal rooster serum	8	0	
Normal rooster serum	8	0	
Normal rooster serum	8	0	

*Age of culture 4–12 hours.
†Challenges of 10 LD_{50} or greater gave 100% mortality.

antiserum or globulin. The globulin from human plasma represented a two- to three-fold concentration of the globulin from the original. Our best passive protection tests were obtained with the rabbit antiserum and human globulin.

Occasionally rabbits and roosters were encountered which possessed anticapsular antibodies prior to injection. Although such animals were called "normal" this was scarcely an accurate designation. It was not surprising, therefore, to find that the serum of these animals possessed protective properties of low magnitude. Absorption of these sera with washed, formalin-killed, encapsulated staphylococci removed the protective properties which they initially possessed.

Commercial staphylococcal antitoxin possessed some protective properties, but the protection engendered was not as effective as that engendered by our antiserum prepared against the encapsulated wound staphylococcus. Absorption of antitoxin with washed, formalin-killed, encapsulated staphylococci removed the protective properties. Antitoxin levels, however, were not determined before or after absorption.

Absorption of the antiserum with washed, formalin-killed *S. epidermidis* cells, prepared from at least six strains of non-encapsulated *S. epidermidis*, was without effect on the protective activity of our antiserum. All absorptions were carried out using 0.1 ml of packed staphylococcal cells per 1 ml of serum. The serum–staphylococcus mixtures were incubated at 37° C for 2 hours, then at 6° C in the refrigerator for 5 days.

Soluble material from culture supernatants of the wound strain of *S. aureus* grown in Casamino Acids glycerol broth was vacuum concentrated. After concentration, this material was dialyzed free of salts, reconcentrated and deproteinized according to a method described by Heidelberger, Kendall, and Scherp (13). The material was made 1% with respect to sodium chloride and precipitated with 8 volumes of 95% ethyl alcohol. The precipitate was dissolved in the minimum volume of 1% sodium chloride solution needed and treated with 1 volume of 95% ethyl alcohol. The mixture was allowed to stand overnight at 6° C and then the precipitate was collected. The precipitate was dissolved in deionized water and clarified by centrifugation. The precipitate after centrifugation was discarded and the supernatant was called P_1S. This fraction was used to absorb antibodies from a preparation of human gamma globulin prepared from blood bank plasma. The results of protection tests using the absorbed plasma fraction are shown in Table IV.

TABLE IV

Protective antibodies and their removal from human gamma globulin

Material	Challenge LD_{50}'s	% protection
Unabsorbed gamma globulin, lot A	10	56
Gamma globulin lot A absorbed with 5 mg P_1S/ml	10	10
Unabsorbed gamma globulin, lot B	6	46
Gamma globulin, lot B, absorbed with 100 μg P_1S/ml	6	8
Unabsorbed gamma globulin, lot C	12	73
Gamma globulin, lot C, absorbed with 50 μg P_1S/ml	12	21

NOTE: Lots A, B, and C represent different preparations of human gamma globulin.

All the different amounts of fraction P_1S were effective in removing the protective antibody. The significance of these findings was tested by the Chi^2 test. All the data were significant at the 5% level.

Discussion

Although Lyons (14) reported finding encapsulated *S. aureus* in pus 23 years ago, remarkably little attention was paid to his report, perhaps owing to failure of contemporaries to confirm his findings. Forssman (8) was convinced that resistance to staphylococcal infections depended on antibacterial antibodies other than antitoxins. Elek (5) concluded that staphylococcal toxins may play a role in the pathogenesis of staphylococcus lesions, but that they were probably not the dominant factor, for the minimal pus forming dose of staphylococci in the human was no different in the presence or absence of staphylococcal toxins (6). It had been shown by Smith (18) that in rabbits immunized with staphylococcal toxoid and challenged with live organisms, the presence of circulating antitoxin did not prevent the localization of the organisms.

Gilbert (12) reported the isolation of a mucoid *S. aureus* at autopsy from the heart of a patient who had died of acute gonococcal endocarditis. Gilbert compared this mucoid strain which she designated the "smooth" variant to that of the non-mucoid "rough" strain which dissociated from the original

on agar plates. Using guinea pigs inoculated intraperitoneally, she showed the smooth variant was more virulent than the non-mucoid variant.

Price and Kneeland (16) isolated an encapsulated *S. aureus* after egg passage of a strain from a case of staphylococcal pneumonia along with a type A prime strain of influenza virus. An antiserum against the encapsulated variant elicited a specific capsular reaction when 3-hour broth cultures were used. When the virulence of the encapsulated variant was compared to that of the non-mucoid parent strain, no difference could be detected. Further studies on the encapsulation phenomenon by Price and Kneeland (17) led to their report of the production of specific capsular reactions in 32 out of 39 strains in a collection from pathological and normal sources. Twenty-eight of the 32 strains originated from pathological sources.

Our interest in encapsulated staphylococci was stimulated by the work of Price and Kneeland (16, 17). A possible relationship between encapsulation and virulence in staphylococci similar to that of pneumococci suggested itself. We did not, however, use mice as our experimental host because of their high resistance to staphylococcal infection. Elek (5) condemned most animal experiments with staphylococci as of spurious validity, particularly in the light of his experiments on human subjects (6). We, however, felt justified in studying staphylococcal infections in embryonated eggs for several reasons. Such studies might disclose virulence factors of the organism and resistance mechanisms of the host which could point the way to a better understanding of human disease. Human subjects are not available for all to experiment on (6). We modified the technique of Frappier *et al.* (9, 10) and found LD_{50}'s of 1400 to several million organisms for coagulase positive strains of *S. aureus*. None of the *S. epidermidis* strains killed enough eggs to permit an LD_{50} calculation, even when over 5 million organisms were injected. It may well be that in the pathogenesis of staphylococcal lesions both (*a*) toxigenic properties and (*b*) resistance to phagocytosis play a role. Thus a strain might be capable of resisting phagocytosis because of the presence of a capsule, but incapable of killing the host because of feeble toxigenicity. The enigma of finding a strain of *S. epidermidis* which was encapsulated and showed a specific capsular reaction with *S. aureus* antiserum is difficult to explain. Culturally it was a typical *S. epidermidis*. The capsular reaction may have been due to a shared cell wall antigen, whereas the low virulence of the *S. epidermidis* strain could have been due to its failure to produce some toxic substance necessary for killing the embryos. In an experiment carried out with the encapsulated *S. epidermidis* strain, all the eggs were opened after 5 days' incubation. None had died, yet all yielded Gram-positive cocci when the allantoic fluid was cultured. The capsule may have played a role in survival of the organisms for such a long period. These findings do not necessarily invalidate our hypothesis concerning the role of the capsule in the virulence of staphylococci. Multiple factors may prove to be involved in the process.

The choice of the egg placed certain limitations on the amount of antiserum that could be injected into the egg. The monograph on egg technique by Beveridge and Burnet (3) indicated a dose of 0.05 ml as optimal for intravenous injection in eggs. The protection engendered by the antiserum, while not of

high degree as compared to passive protection tests in mice with antipneumococcal (7) or antimeningococcal (22) serum, was produced when 0.04 ml of serum or globulin was injected. Part of the problem may be due to the difficulty of finding an experimental host for staphylococci as sensitive as the white mouse for pneumococci and meningococci. In most instances 0.04 ml of antiserum would protect over half the eggs against 5 to 20 LD_{50} doses of encapsulated staphylococci. Our best results in the protection test were obtained with young cultures, possibly due to the liberation of capsular material which was capable of combining with and removing antibody from the system in older cultures.

Our quest for a suitable sample of human plasma for use in the coagulase tests led us to test the ability of human plasma samples to produce a specific capsular reaction with our encapsulated wound staphylococcus. We found over 40% of the samples tested produced a specific capsular reaction with our "wound" strain. The human globulin used in the protection tests was prepared from these samples. Sonea, Frappier, and Borduas (19) and Sonea, Borduas, and Frappier (20) reported a protective effect of human gamma globulin in an experimental staphylococcal infection in mice if gamma globulin were given intraperitoneally at least 2 hours prior to intracerebral challenge with living staphylococci.

It is our desire to study further the role of anticapsular antibodies in resistance to staphylococcal infection by isolation of the capsular substance from culture supernatants of the encapsulated staphylococcus. We shall try to absorb the protective properties from the antiserum, using the capsular substance, and at the same time employ the same capsular substance to inhibit the specific capsular reaction in vitro. If the same material inhibits the specific capsular reaction and absorbs the protection from antiserum, a good argument for the role of anticapsular antibodies in resistance of the host to staphylococcal infection will exist insofar as our system is concerned.

In later papers we shall report the incidence of anticapsular antibodies in healthy humans and describe the isolation and characterization of the capsular substance.

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SUPPRESSION AND STIMULATION OF MYCELIAL GROWTH OF PHYTOPHTHORA CRYPTOGEA BY CERTAIN THIAMINE-REQUIRING AND THIAMINE-SYNTHESIZING BACTERIA[1]

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Abstract

Preculturing a synthetic medium with bacteria of the genus *Arthrobacter* reduced growth of *Phytophthora cryptogea*. The growth reduction appeared to be due chiefly to utilization of thiamine by the bacteria. However, there was some evidence that these bacteria also removed other fungal nutrients from the medium. When the bacteria were streaked on synthetic agar media, radial growth of the fungus on the same plates was reduced only when the thiamine concentration was low. Certain other bacteria stimulated mycelial growth of *P. cryptogea* in a thiamine-deficient medium by producing either thiamine or thiamine-like substances.

Introduction

Since *Phytophthora cryptogea* Pethyb., cause of a root disease of alfalfa (2), requires thiamine for growth (3), a study of the effect of competition for and synthesis of this vitamin by other microorganisms was initiated prior to further studies of the ecological significance of nutrition on its existence in soil. There are several reports giving both direct and indirect evidence that thiamine may be either synthesized or used by soil microorganisms and therefore could conceivably be an important object of competition. Rao *et al.* (11) reported that culture filtrates of *Fusarium vasinfectum* Atk. stimulated growth of the thiamine-requiring *Piricularia oryzae* Br. et Cav. when added to a thiamine-free medium. Also Lochhead showed that thiamine was synthesized by some soil bacteria (5), but that others were auxotrophic for this vitamin (8). The suggestion (7) that "growth factor effects could well be as important as antibiotic activity in affecting the microbial equilibrium" in soil appeared worthy of more investigation.

The reduction of growth of *P. cryptogea* by some thiamine-requiring bacteria and the stimulation of the fungus by thiamine-synthesizing bacteria are described in this report.

Methods and Materials

The methods for growing the fungus were essentially the same as those described in a previous report (3) on its nutrition. The synthetic medium consisted of: sucrose, 15 g; *l*-asparagine, 2.0 g; $MgSO_4.7H_2O$, 0.1 g; $FeSO_4.7H_2O$, 0.001 g; KH_2PO_4, 0.43 g; K_2HPO_4, 0.30 g; $CaCl_2.2H_2O$, 0.01 g; a minor element mixture to give 1 p.p.m. of Zn and 0.02 p.p.m. of Cu, Mo, and Mn; and doubly distilled water to 1 liter. Thiamine concentrations are given in the text. Sucrose was added before autoclaving at 15 lb for 20 minutes since it was

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found that this could be done without reducing growth of the organisms tested. The reaction of the medium was adjusted to pH 6.3–6.5 with *N* NaOH. The fungus was grown for 10 days in 10 ml of medium in 50-ml Erlenmeyer flasks on a shaker. In experiments with *Arthrobacter* spp., the medium was further fortified with the following vitamins (μg/liter): B_{12}, 2; biotin, 1; and folic acid, 100. Solid media were prepared with 15 g/liter of Difco Purified Agar.

Thiamine-requiring soil bacteria selected for this study were: *Arthrobacter aurescens*, *A. pascens*, *A. tumescens*, and *A.* sp. No. 824; other unidentified bacteria known to synthesize thiamine were designated BR 80, NR 49, Sm, and Lop. The *Arthrobacter* spp., obtained originally from soil, and NR 49 and BR 80, isolated from the rhizosphere of flax plants, were stock cultures maintained by the Microbiology Research Institute, Canada Department of Agriculture, Ottawa, Ontario. Sm and Lop were isolated from a contaminated but vigorously growing culture of *P. cryptogea* in a thiamine-deficient medium. The bacteria were maintained on a Penassay Base Agar medium (Difco Antibiotic Medium 2) containing 50% soil extract. Bacterial inoculum (a distilled water suspension) was used at the rate of 1 drop per flask. *P. cryptogea* inoculum consisted of a 3-mm disc of synthetic agar and mycelium per flask. Controls in each experiment showed no fungal response to traces of thiamine that might have been introduced with the inoculum. In experiments involving growth on agar medium plates were inoculated with bacteria by streaking. Two days later a 3-mm disc of *P. cryptogea* was placed 40 mm from the bacterial streak. For certain tests the liquid medium in which bacteria had grown was freed of bacterial cells by centrifugation (cell-free media).

Results

Reduction of Growth of P. cryptogea by Arthrobacter spp.

In the first experiment the four *Arthrobacter* cultures which required thiamine were grown for 3 days on a shaker. The synthetic medium (altered to contain 15 g/liter of glucose as carbon source) contained 75 μg of thiamine/liter. A portion of each culture was then centrifuged to remove the bacterial cells. To determine if the bacteria had utilized some of the original 75 μg/liter of thiamine, an additional 75 μg/liter was added to an aliquot of the actual culture and to a portion of cell-free culture fluid to provide an excess of the vitamin for the growth of *P. cryptogea*. The reaction of each medium was readjusted to pH 6.5 followed by reautoclaving. A negative control medium which contained no thiamine was not included since previous experiments showed that little or no growth of *P. cryptogea* occurred in the absence of thiamine (3).

The mycelial weights on the various media in experiment 1 are shown in Table I. It is apparent that the fungus grew poorly on the medium in which the bacteria had been cultured. The addition of excess thiamine to media in which *A. aurescens* or *A.* sp. No. 824 had grown had no effect on growth of *Phytophthora*, suggesting that factors other than lack of this vitamin were causing inhibition. In the medium precultured with *A. tumescens*, addition of thiamine increased the growth of *P. cryptogea* substantially, and on the medium precultured with *A. pascens* only slightly. Removal of bacterial cells further

reduced the growth of *P. cryptogea* in both thiamine-fortified and unfortified media, suggesting that essential nutrients other than thiamine were removed with the cells.

In experiment 2 the original medium on which the bacteria were precultured was altered to include per liter only 1.0 g of sucrose and 30 μg of thiamine, which was insufficient for maximum growth of the fungus in these experiments. The nitrogen source was changed to 0.5 g/liter of vitamin-free casein hydrolyzate (Nutritional Biochemical Co.) since it appeared to be slightly more

TABLE I

The effect of thiamine*-requiring *Arthrobacter* spp. on growth of *Phytophthora cryptogea*

Bacteria cultured on medium	Weight of fungus mats and final pH of the medium							
	Expt. 1				Expt. 2			
	No excess of thiamine		Plus excess thiamine		No excess of thiamine		Plus excess thiamine	
	mg	Final pH	mg	Final pH	mg	Final pH	mg	Final pH
A. tumescens								
(including cells)	53	7.8	70	8.0	2	4.7	87	5.0
(cell-free)	5	4.9	33	5.3	1	4.9	76	5.1
A. pascens								
(including cells)	6	5.2	14	5.3	2	5.2	85	5.2
(cell-free)	4	5.6	6	5.2	2	4.6	73	5.3
A. aurescens								
(including cells)	0	6.5	0	6.8	2	4.5	87	5.5
(cell-free)	0	6.0	0	7.0	2	4.7	71	4.9
A. sp. No. 824								
(including cells)	24	6.0	28	5.9	5	4.4	106	5.1
(cell-free)	17	5.9	25	6.0	3	4.6	82	5.3
None								
Not centrifuged	—	—	80	7.1	47	4.2	108	5.0
Centrifuged	—	—	—	—	30	4.4	80	5.3

*The original medium contained in experiment 1, 75 μg/liter, and in experiment 2, 30 μg/liter of thiamine.

favorable for bacterial growth than *l*-asparagine. Previous experiments indicated that casein hydrolyzate was also an excellent source of nitrogen for *P. cryptogea* (3).

After 3 days of bacterial growth, the media were fortified by the addition of all the components (except thiamine) of the complete synthetic medium. This was done in an attempt to prevent a deficiency of any component other than thiamine. As in experiment 1, half of each of the media was centrifuged to remove the bacterial cells (cell-free). Portions of these media were further fortified with an excess of thiamine (50 μg/liter) to determine whether or not the original 30 μg/liter had been utilized by the bacteria.

The growth of *P. cryptogea* in experiment 2 in each of these media is shown in Table I and was typical of that obtained in three similar experiments. The greater amount of fungal growth in the fresh (uninoculated) synthetic

medium (containing the extra 50 μg/liter) than in the fresh media containing only 30 μg/liter indicated that the latter quantity was insufficient for maximum growth. However, growth of the fungus was reduced to almost nothing in the low-thiamine media on which bacteria had been cultured, compared to approximately 81% of normal where extra thiamine was added. The failure of the fungus to reach 100% growth on the media precultured with bacteria (except *A.* sp. 824) indicated that nutritional factors other than thiamine might also be involved to some extent. It is noteworthy, however, that in this experiment the precultured media, when fortified with nutrients after growth, did not induce marked or complete inhibition such as that observed for *A. aurescens* in experiment 1 (Table I). This evidence favors the hypothesis that a nutrient deficiency rather than toxin production reduced the growth of *P. cryptogea.*

In two other experiments each of the bacteria and *P. cryptogea* were inoculated opposite each other on plates of sucrose–*l*-asparagine agar. When the concentration of thiamine in the medium was high (2000 μg/liter) *P. cryptogea* grew toward the bacterial streaks with not more than a slight zone of inhibition. However, when thiamine was reduced to a low concentration (10 μg/liter), a distinct zone of inhibition (most marked with *A.* sp. No. 824, Fig. 1) was noted. This experiment gave further evidence that utilization or inactivation of thiamine by the bacteria rather than toxin formation reduced the growth of *P. cryptogea.*

Effect of Synthesis of Thiamine or Thiamine-like Substances by Bacteria on Growth of P. cryptogea

When either thiamine-containing or thiamine-deficient media were inoculated with *P. cryptogea* and thiamine-synthesizing bacteria at the same time, little growth of the fungus occurred. This appeared to be due at least in part to the longer lag period of *P. cryptogea* compared to that of the bacteria. In other experiments growth of *P. cryptogea* occurred on the glucose–asparagine (thiamine-deficient) medium when the bacteria were first grown in it for 72 hours prior to reautoclaving and inoculation with the fungus. For instance, the fungus produced only 1 mg of mycelium on the thiamine-deficient medium, compared to 11 mg on a reautoclaved cell-free culture and 18 mg on a similar medium containing bacterial cells of NR 49. In another experiment, when a

TABLE II

Stimulation of mycelial growth of *P. cryptogea* on thiamine-deficient media in cultures inoculated with thiamine-synthesizing bacteria

	Weight of fungus mats and final pH of medium			
	Thiamine-deficient medium		Thiamine-containing medium*	
Bacterium	mg	Final pH	mg	Final pH
Sm	65	7.5	80	7.4
Lop	19	4.9	73	7.8
Check (no bacteria)	2	4.7	72	7.8

*Differences are not significant.

PLATE I

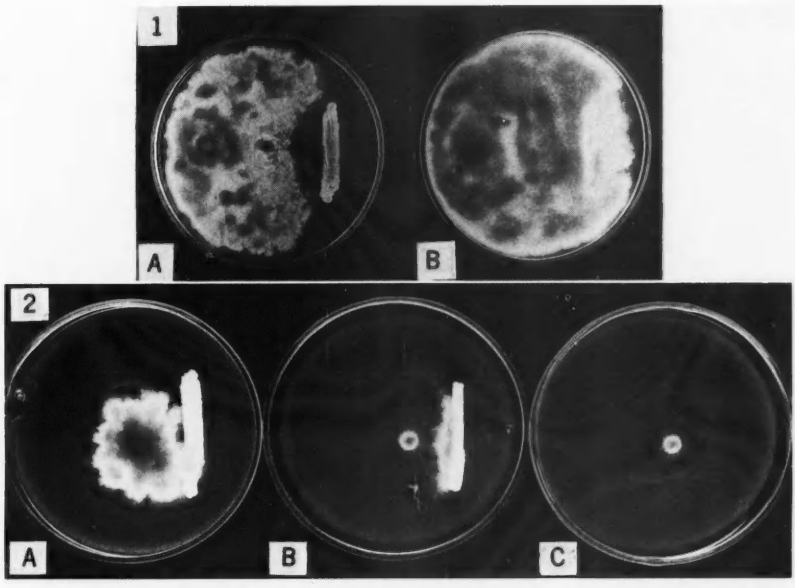

FIG. 1. Effect of a thiamine-requiring bacterium, *Arthrobacter* sp. No. 824, on *P. cryptogea*. (A) Restricted growth of fungus near bacterium on a sucrose-asparagine medium containing only 10 μg/liter of thiamine, and (B) unrestricted growth of the fungus on the same medium containing 2000 μg/liter of thiamine.

FIG. 2. Effect of a thiamine-synthesizing bacterium, Sm, on mycelial growth of *P. cryptogea*. (A) Unrestricted growth of both organisms on medium containing 50 μg/liter of thiamine. (B) On thiamine-deficient medium, good fungal growth limited to vicinity of bacterial streak. (C) Very sparse fungal growth on a synthetic medium containing no thiamine.

reautoclaved thiamine-deficient medium on which the bacterium BR 80 had grown was included as one-third of a synthetic medium and inoculated with *P. cryptogea*, 15 mg of mycelium was harvested compared with 5 mg in the thiamine-deficient medium and 71 mg in the thiamine-containing fresh medium. These growth increases were small but highly significant and suggested that *P. cryptogea* utilized thiamine synthesized by these bacteria. In further experiments in which a thiamine-free sucrose–*l*-asparagine medium was inoculated with *P. cryptogea* and reinoculated 4 days later with the bacterium Lop or Sm, a marked growth stimulation occurred with the latter and appreciable growth with the former (Table II). Culturing the Sm bacterium on a similar thiamine-deficient agar medium resulted in appreciable growth of *P. cryptogea* in the region of the bacterial streak (Fig. 2).

Discussion

Although thiamine was shown to be one of the specific organic factors influencing growth of *P. cryptogea* (expt. 2, Table I), addition of this vitamin to media precultured with bacteria (*A. aurescens*, expt. 1, Table I) but not refortified with the other nutrients, did not result in growth of the fungus. In a completely synthetic agar medium no evidence of toxin formation by the *Arthrobacter* spp. was noted. Since the final pH of the medium precultured with *A. aurescens* following inoculation with *P. cryptogea* was 6.5, pH was probably not a factor. Thus it appears that certain of these bacteria can deplete the medium of one or more nutrients which are essential for growth of the fungus. This aspect of the nutrition of *P. cryptogea* requires further study.

Since growth of *P. cryptogea* was reduced through competition for thiamine by *Arthrobacter* spp. and increased by certain thiamine-synthesizing bacteria, it appears that further studies of competition for thiamine between such heterotrophic fungal plant pathogens as *Phytophthora* spp. and other soil microorganisms might be warranted.

Although thiamine has been detected in soil by other workers (4, 12), it has been found that neither 1:1 soil solutions nor soil (samples from both Ontario and California were added to a thiamine-free synthetic medium prior to autoclaving at concentrations as high as 100 g/liter) supported the fungus in the absence of added thiamine (3). If thiamine is relatively unavailable in soil, competition for this vitamin could be of importance to the existence of the fungus in soil. Morton and Stroube (10) showed that a high percentage of fungi, bacteria, and actinomycetes from soil stimulated growth of *Sclerotium rolfsii* Sacc. in vitro by synthesis of thiamine; however, they discounted the significance of this factor because they were unable to increase the incidence of disease caused by the fungus on soybean plants by addition of certain thiamine-synthesizing organisms to soil. Since thiamine may not influence pathogenicity per se, further studies on other aspects such as persistence of the fungus in soil as influenced by thiamine-producing organisms might be of interest. Because growth factor concentrations in soil are subject to considerable variation as the microbial equilibrium fluctuates (6, 13) experimental assessment of the role of thiamine-synthesizing microorganisms in soil may be quite complex and would necessitate prevention of aerial as well as soil-borne microbial contaminants.

The organic nature of thiamine implies that its existence in soil is undoubtedly influenced by microbial action. This vitamin was once thought to be elaborated entirely by plant roots (14, 15), but recent evidence which shows a predominance of thiamine- and other growth factor-synthesizing organisms in the vicinity of plant roots, suggests that thiamine as well as other vitamins are elaborated chiefly by microorganisms in the rhizosphere. Lochhead and Cook (9) have also shown that the percentage incidence of bacteria capable of synthesizing growth factors was greater on the roots of the susceptible Novelty variety of flax than on the resistant Bison. This finding points "to circumstances that favor a greater accumulation (or 'turnover') of vitamins and related substances at the root surfaces of susceptible plants." It would appear, therefore, that a study of the growth factor relationships in soil as they apply to pathogenicity of plant parasitic fungi and to general ecological consideration would be a fertile though complex subject for future work.

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NOTES

USE OF CATALASE IN THE CULTURE OF VIBRIO FETUS*

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Many workers have found that a large inoculum is usually needed to initiate growth of a culture of *Vibrio fetus*. A similar requirement has been shown by other species of bacteria and by tissue cells (1–4). This study was made to determine whether catalase added to *Vibrio* culture media would reduce the size of the minimum inoculum needed for proliferation in vitro and, thus, would facilitate primary isolation of the organism.

Four catalase-positive strains of *V. fetus* isolated from bovine fetuses, three from Ontario and one from Colorado, were used for these tests. Cells were grown in a 2.8% solution of sterile Brucella broth (Albimi Laboratories Inc., New York) at 37° C under 10–20% CO_2 for 24–30 hours, harvested near the peak of the logarithmic growth phase, washed once in broth, and resuspended in broth. Bacterial counts were estimated by comparing the suspensions with "Wellcome" opacity tubes (Burroughs Wellcome and Company, London) and using the figure of *Vibrio comma*. Doubling dilutions were made so that the final concentrations used for inoculation were 56 million to 0.9 million cells per ml.

Tests were carried out in broth and on plates as follows:

1. Flasks containing 9.7 ml of Brucella broth, 0.1 ml of freshly prepared 1:400 w/v sterile aqueous solution of crude beef liver catalase (Nutritional Biochemicals Corp.), and 0.2 ml of the inoculum dilution were incubated as above. Controls using sterile distilled water in place of the catalase solution were inoculated and refrigerated during the incubation time.
2. Plates prepared by mixing 19.7-ml volumes of sterile Brucella agar with 0.1-ml volumes of catalase 1:400 were inoculated with 0.2 ml of each inoculum dilution.
3. Sheep blood agar plates in place of Brucella agar were prepared as in test 2 and incubated 48 hours.
4. Catalase, to give a final concentration of 1:1200, was added to each inoculum dilution and plated on Brucella agar plates.

The results of these tests were determined by measuring the opacities of broth and saline suspensions in nephelos units on a Coleman Nepho-Colorimeter (model 9). Saline suspensions were prepared by washing the growth from Brucella agar plates, centrifuging, and resuspending the cells in 10 ml saline solution. Table I gives the nephelometric readings of the test and control series for strain 2743, and indicates a 64-fold reduction in minimum inoculum required to initiate growth in broth with catalase, and a 16-fold reduction when catalase was added to Brucella agar. The other strains showed a similar trend.

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TABLE I

Effect of catalase on growth of *Vibrio fetus*, strain 2743

	Growth (measured in nephelos units)			
	Broth		Brucella agar	
Inoculum (million cells/ml)	1:40,000 catalase	No catalase	1:80,000 catalase	No catalase
56	170	130	500	300
28	48	0	420	312
14	86	0	360	—
7	2	0	198	2
3.5	2	0	336	2
1.8	3	0	—	2
0.9			255	2

Similar results were obtained when catalase was mixed with Brucella agar, and when mixed with the cell suspension at time of plating on Brucella agar.

There was no observable increase in growth on blood plates with added catalase when compared with plates without catalase. This would seem to suggest that blood cells supply adequate catalase to promote growth of a small inoculum.

The results of these tests support the conclusion that catalase, added to some culture media not containing blood, significantly reduces the number of *V. fetus* cells needed to initiate growth.

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LYSOZYME AND THE PRODUCTION OF OSMOTIC FRAGILITY IN ENTEROCOCCI

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Although Abrams (1) has prepared protoplasts from the 9790 strain of *Streptococcus faecalis* by the use of lysozyme, the enterococci are considered generally refractory to the action of this enzyme (7). Recently Bleiweis and Zimmerman (3) have reported that, while use of a phage associated lysin produced protoplasts from *Streptococcus faecalis* var. *liquefaciens*, use of a lysozyme–penicillin technique produced forms with partial osmotic fragility but still possessing cell wall components (i.e., spheroplasts).

For over a year lysozyme has been used in this laboratory to produce osmotically fragile bodies from *Streptococcus faecium* HF8AG. Examined by phase microscopy, they appear as discrete spheres rather than as the elliptical, paired cells characteristic of the untreated organism. Upon removal from hypertonic solution to distilled water these bodies undergo immediate lysis, yielding a clear, opalescent solution. Examined by the method of Dische (6) for methylpentose, they give a negative test, suggesting the absence of rhamnose, a methylpentose component of the cell wall in streptococci (8); moreover, treatment with boiling 2 *N* H_2SO_4 for 3 hours, followed by neutralization, ion exchange desalting, and paper chromatography—a procedure that readily detected rhamnose in whole cells—failed to reveal rhamnose in the osmotically fragile forms. Thus, while confirmatory evidence for the lack of residual cell wall might be obtained by electron microscopy, its apparent absence thus far makes it likely that they are true protoplasts (4).

Twelve enterococcus species and varieties have been surveyed for lysozyme sensitivity under the conditions applied to *S. faecium* HF8AG (the cultures were isolated and identified by Mr. Paul Trainor, Graduate Research Assistant of the Department of Bacteriology, University of New Hampshire). The cultures were grown in the basal medium of Chesbro and Evans (5) for testing the alkaline growth capacity of enterococci, except that the pH of the medium was not adjusted to 9.6 after addition of the sterile K_2HPO_4 and Na_2CO_3 (final pH of the medium : 9.4). A second lot of the basal medium was prepared in which 0.1 *M* $NaHCO_3$ was substituted for the 0.05 *M* Na_2CO_3 of the first lot, and the pH was adjusted to 7.4. Screw cap tubes of the media were inoculated with 0.1% of their volume and incubated for 18 hours at 35° C. At the end of this time the cultures were centrifuged, washed, and suspended in buffered 0.4 *M* sucrose (1) to 1/10th their growth volume. Two-tenths of a milliliter of this suspension was withdrawn to 9.8 ml

TABLE I

Osmotic fragility developed in enterococci by exposure to 180 μg lysozyme/ml cell suspension for 65 minutes

Culture		Percentage of initial turbidity in distilled water	
		pH 9.4 growth medium	pH 7.4 growth medium
S. faecalis var. *liquefaciens*	1	100	100
S. faecalis var. *liquefaciens*	6	100	100
S. faecalis	14	100	100
S. faecalis	19	95	100
S. faecalis var. *liquefaciens*	4	100	64
S. faecium	6	76	87
S. faecalis	5	42	68
Biotype*	27	14	36
Biotype	26	11	75
S. faecium	3	10	76
S. faecium	HF8AG	10	19
Biotype	30	08	23
S. faecium	1	–†	17

*Biotypes are distinguished from *S. faecalis*, and its varieties, and *S. faecium* by fermentation, reduction, and hemolytic reactions; and by gelatin liquefaction (2).
†Culture grew and lysed in the growth medium.

of distilled water and the optical density of the diluted suspension determined at 540 mμ. Sufficient lysozyme to yield 180 μg per ml of cell suspension was then added to the samples, and they were incubated at 37° C for 65 minutes, after which a second 0.2 ml was withdrawn and tested for osmotic fragility. Under these conditions *S. faecium* HF8AG was maximally fragile [(optical density of lysozyme treated cells in distilled water) / (optical density of untreated cells in distilled water) was less than 0.15] in 65 to 75 minutes. Cell suspensions held under these conditions in the absence of lysozyme developed no detectable fragility.

Table I compares the turbidities produced in distilled water by the cultures after lysozyme treatment to the turbidities similarly produced by the cultures before treatment, the cultures having grown in the basal medium either at pH 7.4 or at pH 9.4.

TABLE II

Osmotic fragility developed in enterococci exposed to 180 μg lysozyme/ml cell suspension after growth in medium with elevated Na^+ or K^+ content

Culture		Percentage of initial turbidity in distilled water		
		Basal medium (BM)	BM + 1.1 *M* NaCl	BM + 1.1 *M* KCl
S. faecalis var. *liquefaciens*	1	82	100	100
S. faecium	HF 8AG	26	78	66
Biotype	B 30	85	93	100

In the same fashion, Table II shows the development of osmotic fragility in three cultures grown in the basal medium only and in the basal medium containing either 1.1 *M* KCl or 1.1 *M* NaCl. None of these three media contained added carbonate.

The conclusions to be drawn, although derived from a limited number of samples, seem clear cut. The enterococci tested exhibit a spectrum of lysozyme sensitivity, with *S. faecalis* and its variety *liquefaciens* being the most resistant, while *S. faecium* and the biotypes vary from partial to complete sensitivity.

The lysozyme sensitivity of the latter groups is enhanced by carbonate in the growth medium and increased further by an elevated pH in the growth medium, while an elevated Na^+ or K^+ level in the growth medium decreases sensitivity.

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FUNGI ACTIVE IN HETEROTROPHIC NITRIFICATION[1]

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The production of nitrite and nitrate from peptone was described first by Schmidt (7) in the fungus *Aspergillus flavus*, and subsequent studies have revealed that many strains of *A. flavus* are capable of heterotrophic nitrification (2, 3, 4). In addition, Malavolta *et al.* (4) have shown that a culture of *Aspergillus wentii* was able to form nitrite and nitrate in a peptone medium while 10 isolates of *Aspergillus niger* and 2 of *Aspergillus oryzae* were unable to carry out the oxidation. Nitrate production in a medium containing nitrite as a sole nitrogen source has been described in a number of aspergilli (6), in a strain of *A. flavus*, and in several species of *Penicillium* (3).

In an attempt to extend the range of heterotrophic nitrifying fungi, cultures obtained from various sources were grown in the peptone medium described by Eylar and Schmidt (2) and in an ammonium medium made up of two parts. Part A contained sucrose, 8.8 g; $(NH_4)_2HPO_4$, 1.65 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.1 g; $FeSO_4.7H_2O$, 0.001 g; $MnSO_4.4H_2O$, 0.001 g; and distilled water, 500 ml. Part B contained K_2HPO_4, 0.1 g; $Na_2HPO_4.7H_2O$, 26.8 g; NaH_2PO_4, 13.9 g; and distilled water, 500 ml. The two solutions were autoclaved separately to avoid excessive precipitation and were mixed after being allowed to cool. The microorganisms were incubated at 30° C in a static condition. Since cultures of *A. flavus* tend to lose the ability to nitrify after prolonged culture on artificial media (8), all the fungi were inoculated into sterile soil immediately upon receipt and reisolated prior to use.

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TABLE I

Production of nitrite and nitrate by various fungi in peptone and ammonium media

Identity	Culture No.*	Incubation time (days)	Oxidized nitrogen formed (μg N/ml)			
			Peptone medium		Ammonium medium	
			Nitrite	Nitrate	Nitrite	Nitrate
Aspergillus flavus	ATCC1003	20	0.09	0	—	—
A. flavus	ATCC9296	20	0.33	13.0	—	—
A. flavus	ATCC10124	20	0.35	24.5	—	—
A. flavus	ATCC11495	20	0.06	0	—	—
A. flavus	NRRL1779	6	0	0	0	0
A. flavus	NRRL1819	6	0.18	38.5	0	29.5
A. flavus	IZ (15)	6	0	0	0.05	34.5
A. flavus	IZ (1219)	6	0.24	9.8	0.07	25.0
A. flavus	Schmidt	6	0.56	10.5	0.06	40.0
A. flavus	Bush	6	0	0	0	0
Aspergillus oryzae	ATCC9102	20	0.18	4.5	—	—
A. oryzae	NRRL1809	6	0.95	7.0	0.06	9.5
A. oryzae	NRRL1988	6	0	0	0	0
Aspergillus wentii	NRRL1599	6	0	0	0.12	34.5
A. wentii	NRRL1778	6	0	0	0.08	8.0
A. wentii	IZ (1220)	6	1.20	0	0	25.5
Penicillium atrovenetum	SM 683	6	0.23	0	0.10	0

*Origin of cultures

ATCC: American Type Culture Collection, Washington, D.C.
NRRL: Northern Utilization Research and Development Division, U.S.D.A., Peoria, Illinois.
IZ: Dr. J. D. P. Arzolla, Instituto Zimotécnico, Piricicaba, Brazil.
Schmidt: Dr. E. L. Schmidt, University of Minnesota, Minneapolis.
Bush: Dr. M. T. Bush, Vanderbilt University, Nashville, Tennessee.
SM683: Dr. J. H. Birkinshaw, London School of Hygiene and Tropical Medicine.

The results in Table I provide further evidence for the widespread occurrence of the nitrifying ability among strains of *A. flavus*, and indicate that this property may be common to strains of *A. wentii*. It is obvious that some cultures of *A. oryzae* also are capable of oxidizing reduced forms of nitrogen to nitrite and nitrate.

Since β-nitropropionic acid has been implicated in the pathway of heterotrophic nitrification by Schmidt's strain of *A. flavus* (Marshall and Alexander, unpublished data), two fungal cultures known to be able to produce β-nitropropionic acid under certain conditions were obtained and tested for their ability to produce nitrite or nitrate in the media described above. The *A. flavus* strain of Bush *et al.* (1) failed to produce nitrite or nitrate while *Penicillium atrovenetum* SM683 (5) formed small quantities of nitrite but no nitrate in both media. Surprisingly, this culture of *P. atrovenetum* did produce significant amounts of nitrate (13 μg nitrate-N/ml) when grown in the nitrite medium suggested by Hora and Iyengar (3). The ATCC and NRRL cultures listed in Table I were grown in this nitrite medium, but only two strains of *Aspergillus wentii* (NRRL 1599 and NRRL 1778) accumulated nitrate.

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NATURAL INHIBITORS OF CELLULASE

MARY MANDELS, W. HOWLETT, AND E. T. REESE

Stimulated by the discovery of active cellulase inhibitors in *Eucalyptus rostrata* (Jermyn and Youatt, CSIRO, Parkville, Victoria, Australia, personal communication, 1957), and in *Vitis rotundifolia*, the Scuppernong grape (1, 2), we have tested extracts of leaves, flowers, fruits, and twigs of local plants, seeds (germinated and ungerminated), and woods. We find that natural inhibitors of cellulase are of common occurrence (Table I). A few plants contain quite active inhibitors (Table II). The best sources include bayberry, the closely related sweet fern, and a number of tropical woods, including *Eucalyptus rostrata*. We did not have a variety of *Vitis rotundifolia* for testing, but of four varieties of *V. vinifera* and *V. labrusca*, only Baco No. 1 (*V. labrusca*) was active (36 inhibitor units per gram of leaf).

The specificity of three inhibitors was determined for a variety of carbohydrases (Table III). While these plant extracts strongly inhibited most cellulases, a few cellulases are resistant. This variability in response was observed earlier by Bell *et al.* (1) for their natural inhibitor, and by us (6) for such chemical inhibitors as methocel, cellobiose, sodium ethylene bisdithiocarbamate, and iodine. Similar variability due to source of enzyme has been observed for inhibitors of amylase (4), and of β-glucosidase (3). Of the other carbohydrases tested, none was appreciably affected by the three natural inhibitors of cellulase (Table III).

The bayberry inhibitor is soluble in water, methanol, and ethanol, and is generally insoluble in other organic solvents. It is resistant to boiling in water or acid, and to the action of various carbohydrases. It is, however, quickly destroyed by exposure to mild, alkaline conditions at room temperature. The activity can be adsorbed on cellulose, charcoal, or microcel, but has not been successfully eluted. By solvent extraction, a preparation active at 3 parts per million was obtained.

TABLE I

Occurrence of cellulase inhibitors in plants

Plant source	Samples tested	Samples active*
Local collections (Natick, Mass.)	122	26
Seeds	25	0
Woods		
Jesup collection (Harvard)	71	4
Stahel collection (Harvard)	55	7
Krukoff collection (Smithsonian)	125	12
Other	4	1

*Dry samples of plants were ground and extracted in methanol and in water (1 g/10 ml). One unit of inhibitor causes a 50% reduction in the activity of 2 units of cellulase of *Trichoderma viride* (5). Samples, to be considered "active", had to contain at least 12.5 units of inhibitor per gram of tissue.

TABLE II

Most active sources of cellulase inhibitor

Name of plant	Part	Activity in units/gram sample
1. *Comptonia peregrina* (sweet fern)	Leaves	470
2. *Myrica pensylvanicum* (bayberry)	Twigs	285
Myrica pensylvanicum	Leaves	255
Myrica pensylvanicum	Berries	249
3. *Cynometra krukovii*	Wood	210
4. *Ormosia coccinea*	Wood	200
5. *Curatella americana*	Wood	130
6. *Eucalyptus rostrata*	Wood	130
7. *Mimusops* sp.	Wood	126
8. *Micrandra bracteosa*	Wood	125
9. *Stryphnodendron paniculatum*	Wood	100
10. *Moronobea coccinea*	Wood	70
11. *Gaylussacia baccata*	Leaves	59
12. *Polygonum cuspidatum*	Leaves	59

TABLE III

Specificity of natural inhibitors

Enzyme source		Inhibitor source		
		Comptonia	*Myrica*	*Eucalyptus*
	QM	Inhibition %		
Cellulase = β-1,4-glucanase				
Trichoderma viride	6a	75	75	66
Myrothecium verrucaria	460	85	91	70
Streptomyces	B814	93	87	80
Basidiomycete	806	86	68	65
Pestalotiopsis westerdijkii	381	57	33	21
Penicillium pusillum	137g	6	2	15
Amylase = α-1,4-glucanase				
Aspergillus luchuensis	873	0	0	13
β-1,6-glucanase				
Aspergillus quadricinctus	6874	+	+	+
β-1,3-glucanase				
Aspergillus quadricinctus	6874	11	9	9
β-glucosidase = cellobiase				
Almond emulsin		15	0	+
Aspergillus quadricinctus	6874	11	8	13
Pestalotiopsis westerdijkii	381	3	+	+

The presence of cellulase inhibitors in plants parallels the well-established existence of amylase inhibitors (4). The high activity and marked specificity of these inhibitors leads to speculation as to their role in growth, development, and disease resistance.

We are grateful to Dr. Richard Howard of the Arnold Arboretum and Dr. William Stern of the Smithsonian for herbarium specimens of wood; to Miss Pamela Brady for assistance with the screening; and to Mrs. Mildred Hyatt for aid in the identification of local plants.

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ANOMALOUS INFECTIONS BY RHIZOBIUM

R. T. LANGE AND M. ALEXANDER

The biochemical mechanisms by which species of *Rhizobium* infect, induce the formation of root nodules, and enter into the N_2-fixing symbiosis with leguminous plants are poorly understood. There are many lines of evidence which point to a bacterial role and a plant role in the infection and symbiotic processes. Bergersen (3) has recently advanced a most interesting hypothesis for the mechanism of symbiotic fixation of N_2 by the *Rhizobium*–legume association.

On the basis of the studies of Fahraeus and Ljunggren (4, 5), it appears that there is a relationship between a polysaccharide of the microsymbiont and the formation by the specific macrosymbiont of polygalacturonase, and it may be that pectic enzymes are directly concerned with bacterial pensetration into the host's tissues. In an investigation designed to determine whether nodulation by a non-infective *Rhizobium* sp. can occur in the presence of polysaccharides from an infective bacterium, successful infections

outside of established cross-inoculation groups have been achieved, but the data suggest that the anomalous infections arise by a somewhat different mechanism.

Cultures of *Rhizobium* 3F6h9, *Rhizobium meliloti* 3DOh13, and *Rhizobium trifolii* 3D1m31a were grown for 3 weeks with continuous aeration in 4 liters of a mannitol – yeast extract liquid medium (1). These bacterial strains were infective upon and isolated from nodules of *Astragalus sinicus*, *Melilotus alba*, and *Trifolium pratense*, respectively. The cells were removed by centrifugation at 17,000 × *g* for 20 minutes and 10% w/v NaCl added to the resulting supernatant solution. To precipitate a polysaccharide-rich fraction from the culture filtrate, 1 volume of acetone and 1 volume of ethanol were added to each volume of the supernatant. The bulk of the liquid was expressed from the resultant coagulum by mechanical pressure, and the coagulum was then dried, dissolved in water, reprecipitated as before, redried, and dialyzed against distilled water. The final polysaccharide-containing fraction was stored at 4° C until used.

Each of the three legume species was inoculated with each of the three microbial strains in two parallel series. In the first series, the seedlings were inoculated with the rhizobia alone, the inoculum containing approximately 2×10^9 cells. The second series was identical with the first except that the plant received not only the bacteria but also 30–50 mg of the polysaccharide-containing preparation obtained from the *Rhizobium* strain specific for the individual legume in question. In addition, control series containing uninoculated seedlings and uninoculated seedlings treated with the polysaccharide fraction were included. Six replicate tubes were used in all treatments.

In the first series, *A. sinicus* was nodulated only by *Rhizobium* 3F6h9, *M. alba* only by *R. meliloti*, and *T. pratense* solely by *R. trifolii*. The times required for signs of visible nodulation were 20, 10, and 7 days, respectively. None of the three plant species developed nodules when exposed to bacteria of another host–bacterium grouping. Further, seedlings grown in tubes containing no bacteria and those grown in the presence of the polysaccharide-rich fraction remained nodule-free.

Infections were noted outside of the established cross-inoculation groups when the various legumes were exposed to the bacteria in the presence of preparations made from the normally infective *Rhizobium* strains. Thus, each of the three rhizobia nodulated *T. pratense* in 7–11 days when the rooting medium contained the *R. trifolii* preparation. Similar results were obtained for the three bacteria exposed to *M. alba* with the *R. meliloti* fraction in the agar and to *A. sinicus* in the presence of the preparation from *Rhizobium* 3F6h9. Nodule appearance required 9–16 days for *M. alba* and approximately 20 days for *A. sinicus*. For reasons not yet clear, not all of the seedlings developed root nodules. The active materials in these preparations appeared to be rather unstable, and the fractions obtained from *R. trifolii* and *Rhizobium* 3F6h9 rapidly lost activity when stored at 4° C. The preparation from *R. meliloti* was appreciably more stable, however, and the activity persisted for several weeks.

The infection by root nodule microorganisms of plants outside of the typical cross inoculation groups in the presence of suitable bacterial polysaccharides

might be taken to indicate that it is the polysaccharide which is active in the fractions described herein; for example, the specific polysaccharide may serve to stimulate production of polygalacturonase by the appropriate host species, as shown by Ljunggren and Fahraeus (5), and the host specificity of *Rhizobium* spp. may disappear once the polygalacturonase has been generated. That this explanation does not account for the anomalous infection patterns is suggested by the observation that a 24-hour incubation of the polysaccharide-rich fraction with crystalline deoxyribonuclease (Sigma) led to a destruction of the active principle, and no cross infections were noted. Moreover, isolates from nodules produced on aseptically grown *M. alba* by *R. trifolii* cells in the presence of the bacteria-free *R. meliloti* preparation behaved symbiotically like *R. meliloti* strains when tested against a range of hosts.

The results suggest that non-infective rhizobia have been transformed through the agency of the deoxyribonucleic acid of infective strains. Such transformations would cross the commonly accepted species boundaries of members of the genus. Transformation of host specificity of *Rhizobium lupini* by deoxyribonucleic acid from *R. meliloti* has been reported by Balassa (2), who also noted that not all plants developed nodules.

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GENETIC STUDIES OF EFFECTIVE NODULATION IN LOTUS SPP.

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The genetics of nodulation and nitrogen fixation in legumes is still a relatively unexplored field. Nutman (4) described a genetic system of resistance to nodulation in red clover. Williams and Lynch (8) noted a mutation in the soybean which causes resistance to infection and nodulation by *Rhizobium japonicum*. Aughtry (1) reported some studies on the genetic basis of nodula-

tion and nitrogen fixation in *Medicago sativa* and *Medicago falcata*. Nutman (5, 6, 7) made a more extensive study on the genetic basis of effective nodulation in red clover. However, these studies were limited to intraspecific variation in nodulation, and they did not help to elucidate differences in specificity to *Rhizobium* spp. among related species of legumes.

The object of the study reported here was to attempt to determine how interspecific hybrids between *Lotus uliginosus* and *Lotus corniculatus* would react with strains of *Rhizobium* which are specific to either parent. By specificity is meant here that the strains form effective nodules with the host plant. Most strains which nodulate *L. corniculatus* effectively induce formation of ineffective nodules on *L. uliginosus*, and vice versa. For this purpose two *Rhizobium* strains specific for *Lotus* spp. were selected, one specific for *L. corniculatus* and one for *L. uliginosus* (methods are described in Gershon (1961 (3)). Strain 3 forms effective, i.e. nitrogen-fixing, nodules with *L. corniculatus* seedlings, and strain 10 forms effective nodules with *L. uliginosus* seedlings.

L. corniculatus is a natural tetraploid with chromosome number of $4n = 24$. *L. uliginosus* is a diploid having a chromosome number of $2n = 12$. Tetraploid *L. uliginosus* utilized in this study and the F_1 hybrids of $4n$ *L. uliginosus* × *L. corniculatus* were described by Bent (2).

An experiment was set up to study the reaction of the interspecific hybrids with *Rhizobium* strains 3 and 10. For this purpose, seedlings and cuttings of *L. corniculatus*, $4n$ *L. uliginosus*, F_2 and F_3 hybrids of $4n$ *L. uliginosus* × *L. corniculatus* were planted aseptically in an agar medium contained in test tubes (3). Two cuttings each of F_1, F_2, a triploid hybrid ($2n$ *L. uliginosus* × *L. corniculatus*), and the progeny of the backcross of the triploid hybrid to *L. corniculatus* were also planted and inoculated in test tubes. The results are summarized in Table I.

TABLE I

Results of inoculation of the two *Lotus* species and their interspecific hybrids with two *Rhizobium* strains specific to either parent species

Plant	Chromosome sets from *L. uliginosus*	Chromosome sets from *L. corniculatus*	Effectiveness of nodules*			Total number of plants
			Strain 3	Strain 10	Control	
$4n$ *L. uliginosus*	4	0	−(4)	+(4)	−(1)	9
L. corniculatus	0	4	+(8)	−(9)	−(2)	19
F_1 hybrids	2	2	−(25)	+(23)	−(11)	59
F_2 hybrids	2	2	−(48)	+(48)	−(24)	120
F_3 hybrids	2	2	−(79)	+(74)	−(11)	164
F_1 × *L. corniculatus*	1	3	+(2)	+(2)	−(2)	6
Triploid hybrid × F_1	1	2	−(2)	+(2)	−(2)	6

*Number in parentheses denotes number of plants tested. + or − denote effective or non-effective nodulation respectively.

This study brings out two points of interest. (1) The *L. uliginosus* genome has a dominant expression when combined with the *L. corniculatus* genome with regard to specific association with *Rhizobium* strains and effective nitrogen fixation. (2) There is a chromosomal dose effect involved in effective nod-

ulation of the hybrids; one set of *L. uliginosus* chromosomes gives effective association with strain 10 even in the presence of two sets of *L. corniculatus* chromosomes but not in the presence of three sets; two sets of *L. corniculatus* chromosomes fail to permit effective nodulation with strain 3 in the presence of one set of *L. uliginosus* chromosomes, but three sets permit nodulation. All the hybrids of *4n L. uliginosus* × *L. corniculatus* have two genomes of each parent species. They nodulate only with strain 10, which is *L. uliginosus*-specific. The triploid hybrid of *2n L. uliginosus* × *L. corniculatus* has one genome of *L. uliginosus* and two genomes of *L. corniculatus*. It still nodulates with strain 10 of the *Rhizobium*. Progeny of the cross *L. corniculatus* × F_1 possess three genomes of *L. corniculatus* and one of *L. uliginosus*. These plants form effective nodules with both *Rhizobium* strains 3 and 10.

These results and data summarized elsewhere (3) indicate that the differences in specificity of effective association with *Rhizobium* strains between two related legume species may involve a number of genes. It seems that several genes located on different chromosomes of the genome of the parental plant species are involved in the determination of specificity for effective nodulation. These conclusions are supported by the fact that both the F_2 and F_3 generations did not show segregation into two classes of plants, those which did and those which did not form effective nodules with strain 3.

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